

A flight of fancy

Agricultural biotechnology isn't the first promising new technology to become ensnared in trade politics. The same fate buried supersonic commercial air travel — but that time, the boot was on the other foot.

With the breakdown of consultations between the parties last week, it is now clear that the United States will pursue a formal complaint at the World Trade Organization (WTO) against the European Union, over trade in genetically modified crops.

But as transgenic food shudders on the runway amid increasingly heated rhetoric, it is worth taking a look at another promising technology whose commercial life has just met a premature end.

Last month, an Air France Concorde took its final flight to Washington's Dulles Airport, where it will form a prized exhibit at a new Smithsonian aircraft museum. Just in case the curators at the Smithsonian omit to do so, let's recall the role that American protectionism played in Concorde's rise and fall.

When Concorde's working prototype was making its first, awe-inspiring flights 35 years ago, the plane represented a rare technological triumph for the publicly funded, Anglo-French consortium that built it. Boeing had dropped out of supersonic airliner development, scared by its high development costs. The European success wasn't exactly welcomed in the United States with open arms.

American politicians instead fermented public fears about the noise — the supersonic boom — that the aircraft left in its wake as it passed overhead. Environmental regulators ruled that supersonic flight wouldn't be permitted there — killing Concorde's prospects of such lucrative routes as London to Los Angeles. Faced with these rules, no US airlines ordered the jet. The dearth of orders meant vital economies of scale were never realized, in either production or maintenance.

Many factors influenced the demise of the airliner, which British Airways will finally withdraw from service this autumn. And Concorde's noisiness and thirst for fuel hardly commend it in today's environmentally preoccupied world. Nonetheless, there is greater

demand today for fast, relatively expensive air travel than its architects could ever have imagined. Supersonic civil aviation is dying this year, in part because US protectionism nearly strangled it at birth.

Move on 35 years, and see how much — or how little — has changed. "Countries shouldn't be able to erect [trade] barriers for non-scientific reasons," Don Lipton, a spokesman for the American Farm Bureau Federation, said last week. "That's a very important principle in international trade." Tell that to Concorde's engineers, to Mexican sugar-growers, Brazilian steel-makers or anyone else trying to trade freely in what America doesn't want to buy.

The WTO fight comes at a time when European governments are trying to take steps that will allow the cultivation of transgenic crops in the face of public hostility. The Bush administration considers these steps inadequate, and it may be right. But by raising the stakes now, the administration makes it even less likely that European consumers will accept the technology. The administration probably knows this, but values the short-term political gain to be had from 'taking a stand' on the issue above the longer-term benefits that might accrue from letting Europe accept the technology at a pace that public opinion can bear.

The corporations that own the technology can't afford to wait, of course. Monsanto's hopes of building a multi-billion-dollar life-sciences business on it have already flopped, and there is a sense that unless Europe eats its transgenic dinner soon, nervous farmers in other food-exporting countries, notably China and Brazil, will balk at the technology and bring it to its knees.

In that unfortunate event, London's Natural History Museum could perhaps build a memorial exhibit for it featuring a plinth that might equally adorn the Smithsonian's Concorde display. "This technology wasn't bad," it would read. "It died of political hypocrisy." ■

Building bridges

Science and technology agreements between the European Union and Arab countries are a small, but welcome step.

The European Union's (EU's) freshly signed science and technology cooperation agreements with Morocco and Tunisia — soon to be followed by another one with Egypt (see page 906) — are to be welcomed on several grounds.

Research in the Arab world is in a bad way, and the agreements send a hopeful message to those who want to turn the situation around and use science and technology to improve society and spur economic growth (see *Nature* **416**, 120–122; 2002). Also, with immigration from North Africa a hot political subject in Europe, the EU is acting in its own interests if it helps economies in the region to prosper in peace.

In Morocco, researchers are already involved in some 200 EU projects. Most of these are in areas close to the nation's pressing needs, such as management of water resources, transport and agriculture. The EU hopes now to encourage more of the same, by helping researchers to identify potential partners in Europe, and to work through the European Commission's legendary paperwork.

Another potential benefit of the agreements is that they can only assist in building political dialogue in the Middle East. Joint, practical

projects, such as developing methods for the treatment of waste water from the olive-oil industry, can surely improve relations in the region, as can purely scientific ones, such as SESAME, the synchrotron being built in Jordan. In the words of Said Assaf, a scientist from Ramallah in Palestine who is involved in SESAME: "This project is a bridge to peace through science. As Arabs, we realize that acquiring technology is very important for the advancement of our people."

Indeed, science is one of the few areas where constructive dialogue can be maintained between peoples in conflict. The EU–Arab agreements follow on from a renewal of Israel's participation in the EU's sixth Framework research programme earlier this month, and Philippe Busquin, the European research commissioner, hopes these "will pave the way to enhanced dialogue between Arabs and Israelis".

Such dialogue will take time. Israel shares some problems — such as desertification — with its Arab neighbours, but in its research capacity is closer to Switzerland or Sweden than to Egypt or Jordan. And as long as Palestinian scientists work in the shadow of an occupying army, scientific collaboration will remain more of a cherished ideal than a reality. ■

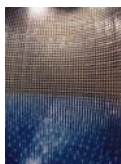




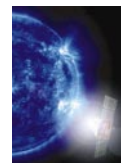
Expansion team
Europe strengthens
research links with
Arab nations
p906



Sour times
Milk project may shut
down firm that gave
birth to Dolly
p907



Digging deep
India plans
underground lab
to study neutrinos
p909



Ray of hope
Sun observation back
on track with fixed
antenna
p910

WHO issues rallying cry to keep fight against SARS on track

David Cyranoski, Kuala Lumpur

The immediate crisis surrounding severe acute respiratory syndrome (SARS) may be subsiding, but most of the key scientific questions about the disease remain open.

These must not be forgotten, participants were told at the first global conference on SARS, held on 17–18 June in Kuala Lumpur, Malaysia. Health officials at the meeting, organized by the World Health Organization (WHO), expressed fears that as memories of the SARS outbreak fade, so too will the determination to answer outstanding questions.

Speakers at the meeting pledged to maintain the research momentum. “We are looking for a treatment; we are looking for a vaccine,” said the WHO’s director general, Gro Harlem Brundtland. “We are looking for some understanding that will keep this from happening again.”

Brundtland called on governments to do more to prepare for a recurrence of the disease. “Whether it is SARS or something else, there will be new threats,” she said. Researchers at the meeting echoed her warnings against complacency, noting that previous pandemics, such as the influenza outbreak in 1918, began with a relatively light first appearance before returning later in full force.

David Heymann, the WHO’s executive director for communicable diseases, called for



Action stations: Gro Harlem Brundtland warns against complacency over infectious diseases.

an international research agenda on diagnostic testing, antiviral drugs and vaccines for SARS. “This is a pivot point from a short-term strategy, to a long-term strategy,” he said. But the WHO, unsure of what support it will get from member countries, has been unable to

set concrete deadlines for such a strategy.

Health officials hope that the unprecedented international effort to contain and investigate SARS will act as a useful model for handling future outbreaks of other diseases. The WHO wants to build on the effort by

Biologists disappointed as NIH budget falls flat

Erika Check, Washington

The recent, rapid expansion of the US National Institutes of Health (NIH) seems to be over — at least for now.

On 19 June, the House of Representatives subcommittee that sets spending for the biomedical research agency recommended a budget increase of just over 2% for next year. That would take the NIH budget to \$27.7 billion — similar to the level proposed by President Bush back in February, but not enough for research advocates.

“This is really bad news,” says Steven Teitelbaum, a cell biologist at the

Washington University at St Louis, Missouri, and president of the Federation of American Societies for Experimental Biology (FASEB). “I have lost a lot of sleep over this — there’s no doubt this is a crash landing for the NIH.”

Congress has traditionally supported strong spending on biomedical research — it was its appropriations subcommittees that implemented a doubling of the NIH’s budget from \$13.6 billion in 1998 to \$27.3 billion this year.

Lobby groups had appealed to Congress to provide more money to maintain the momentum from the doubling. According to

FASEB, the proposed numbers will allow the NIH to fund just 344 new grants next year, of which only 21 will support research unconnected to bioterrorism. That is because almost all of the proposed budget will be eaten up by multi-year grants that were awarded while the NIH was growing.

A Senate subcommittee will deliver its NIH budget this week, and the two versions will then be reconciled to give a final figure. But research lobbyists don’t expect the Senate to offer much more cash than the House — and they fear that the NIH could now face several years of essentially flat budgets. ■

agreeing international standards for important aspects of SARS research, such as treatments and diagnostic tests. The latter will be especially important for ruling out SARS when other respiratory diseases occur. "It will be quite a challenge," says Klaus Stöhr, project leader for the WHO's influenza programme.

There are already dozens of tests that can identify the virus that causes SARS, but they are not yet sensitive enough to confirm infection during the early stage when patients may be passing the virus on to others. Some researchers have claimed accuracies as high as 95% for tests that can be done earlier, but so far "there have been no standards for evaluating the tests," says Christian Drosten, a virologist at the Bernhard Nocht Institute for Tropical Medicine in Hamburg, Germany. And there is surprisingly little agreement on what works and what doesn't. "The diagnostic tests are a real mess right now," says one disgruntled researcher, who didn't want to be identified.

One obstacle is the lack of standardized SARS samples. But the WHO has now persuaded Hong Kong to make samples, including blood and sputum, available at shipping cost to both private and public researchers. Stöhr adds that the WHO is starting to distribute "gold standard" viral samples against which the WHO's collaborating centres can assess the performance of their tests.

But Brundtland and others voiced concern that the WHO's efforts alone cannot keep research momentum going as the number of SARS patients drops off. "The WHO isn't made of money," she says. "Scientists and politicians in individual countries have to continue their efforts."

Visa rules leave US colleges facing semester of discontent

Geoff Brumfiel, Washington

Immigration rules will wreak havoc at US universities when the academic year begins this autumn, administrators are predicting.

The leaders of four groups representing most US university and college administrators — including the Association of American Universities (AAU), which speaks for 50 top research schools — outlined their concerns in a letter to US secretary of state Colin Powell on 17 June.

They warned that visa delays could keep many foreign students and academic staff out of the country, causing "classes to be cancelled, and educational and research opportunities to be lost".

The administrators are particularly worried about an instruction issued by the state department on 21 May, which they fear will dramatically increase the number of visa applicants who must undergo a face-to-face interview with consular officials.

At present, about 40% of all visa applicants are called in for interview, according to Barry Toiv, a spokesman for the AAU. But under the new instruction, embassies and consulates must interview virtually every applicant aged between 16 and 60 — without any increase in resources. "It will slow down a process that is already very cumbersome," Toiv says.

The instruction will pile more pressure on a system already under great strain, university officials say. Many students and researchers have had their visits to the United States delayed by weeks or months by an inter-agency review process set up to screen for



terrorists (see *Nature* 422, 457–458; 2003).

In addition, a computer database at the Department of Homeland Security, which will track foreign students and visiting scholars, is expected to start working in time for the new academic year, creating more headaches for the universities.

Debra Stewart, president of the Council of Graduate Schools and one of the letter's signatories, predicts that some students and staff will head for other countries such as Britain, France and Australia, in the face of lengthening delays.

State department spokeswoman Brooke Summers says that the agency is working to alleviate the delays. "We're increasing our staff and doing all that we can," she says, adding that the current environment requires extra security in the visa-screening process.

European deal aims to attract Arab scientists

Nicola Nosengo, Munich

The European Union (EU) is taking steps to attract more scientists from Arab countries into its Framework programme on research.

Later this week, European research commissioner Philippe Busquin will sign fresh agreements on science and technology cooperation with Morocco and Tunisia. A further agreement with Egypt is likely to be reached during the summer, officials at the European Commission say.

The officials hope that the move will boost Arab science, nurture political dialogue in the Middle East, and fend off criticism that the EU discriminates in favour of Israel.

In theory, scientists anywhere in the world can participate in Framework projects that are led by researchers from nations that are full members of the programme. But

scientists in Arab countries are mostly unaware of the opportunity, research administrators say, or may be deterred by the complexity of the application process.

Under the new agreement, researchers in Morocco and Tunisia will be given up-to-date contact information for all of the groups taking part in Framework projects, and will get assistance in making applications to join in.

"A strong connection to European research is essential for science in north Africa," says Youssef Alouane, president of the University of Tunis-El Manar, one of Tunisia's leading universities. "We are very pleased with these agreements."

The agreements will be signed just two weeks after Israel's full participation in the Sixth Framework Programme was

confirmed. Under an agreement in place since 1998, Israel pays its share of Framework's overall cost and participates fully in the programme.

Maria Kayamanidou, an official at the European Commission, hopes that the new agreements will also boost cooperation between Arab and Israeli researchers. "Israel and the surrounding countries have a strategic interest in cooperating for research," she says. "We try to provide them with a framework to do that."

But some continue to view the agreement with Israel — which includes the Palestinian territories — as discriminatory. "The EU scientific cooperation policy has no significant impact on our region," says Imad Khatib, the Secretary General of the Palestine Academy for Science and Technology.

Photos stop as Landsat 7 defies engineers

Tony Reichhardt, Washington

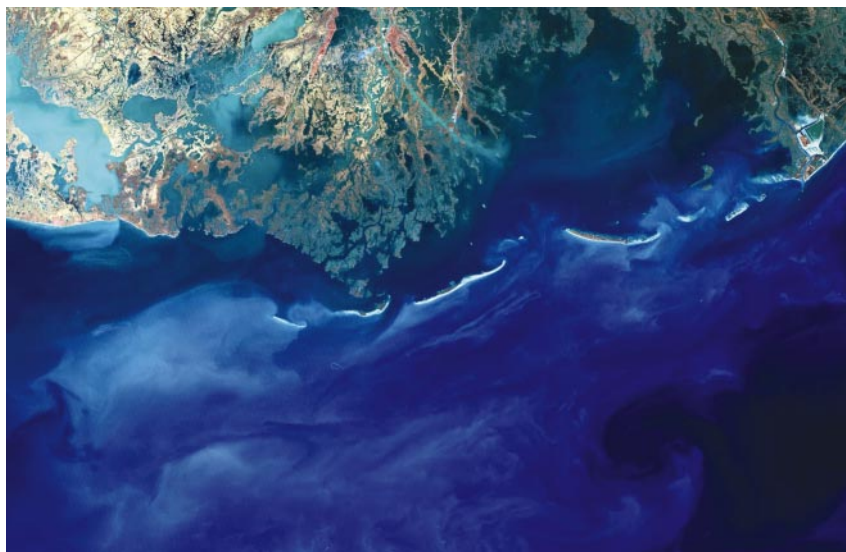
Engineers are struggling to fix a problem with the Landsat 7 remote-sensing satellite that has left global-change researchers without their primary source of new large-scale images of the Earth.

Project managers for the US Geological Survey, who manage the satellite from NASA's Goddard Space Flight Center in Maryland, have yet to fully understand the situation. The fault, which first occurred late last month, lies with the Scan Line Corrector, a device that compensates for Landsat's motion and stops pictures becoming blurred. Without it, about 25% of each image is severely degraded.

Bruce Quirk, the Geological Survey's chief scientist for remote-sensing systems, says it may take two more weeks to find out whether the failure is electrical or mechanical. An electrical fault would be preferable, as the satellite carries back-up electrical systems.

But engineers could find new ways of creating useable images if the failure is mechanical, adds Quirk. Data near the centre of each image are less affected, and it should be possible to provide equivalent coverage by combining multiple images of a ground target — although the process would increase the time needed to create undistorted scenes.

Regular photography has been suspended, a blow to the remote-sensing researchers who used it. The satellite and its predecessors, each government-run, have been taking the same kind of image for 31 years, allowing users to track changes over time. Landsat 7 normally collects 250 large-scale images, of 183×170 kilometres each, every day.



Picture this: researchers rely on Landsat's images, such as this one of silt in the Gulf of Mexico.

The problem comes just as Landsat's privatization is being considered. Congress and the Bush administration would like to see the next satellite owned and run by the private sector, which would sell the data to scientists and other users (see *Nature* **419**, 328; 2002).

But one of two companies expected to bid for the contract — DigitalGlobe of Longmont, Colorado — withdrew earlier this year, saying the venture was too risky commercially. The government insists on keeping image prices low enough for researchers to afford, and the market — mainly federally funded scientists — is not large.

That left Resource21 of Denver, Colorado,

as the only company known to be in the running. NASA officials won't say whether they have received other bids, but the contract award, planned for this month, has been delayed, probably until late summer.

Thomas Lillesand, director of the Environmental Remote Sensing Center at the University of Wisconsin at Madison, says that trying to increase Landsat usage is a chicken-and-egg problem. The market for images should be growing as new image-analysis tools become available, but users won't commit themselves unless "we can get sufficient numbers of satellites up there that we can depend on". ■

Dolly firm in trouble after transgenic milk fails to flow

Jenny Hogan, London

The company that helped create Dolly may follow the cloned sheep to an early grave.

PPL Therapeutics announced on 18 June that its leading project, an attempt to harvest therapeutic proteins from the milk of transgenic sheep, was being put on hold. About half of the Edinburgh-based firm's 200 employees will lose their jobs, and the company itself could fold, some analysts say.

When PPL was established in 1989, the company hoped to clone flocks of genetically modified sheep and cows, and milk them for therapeutic proteins — and for money. But the firm has been forced to sell off several transgenic projects in the past few years. Its share price, which exceeded £4 (US\$6.68) in the mid-1990s, is now about 6 pence.

The latest setback concerns plans to

develop a protein treatment for lung diseases such as hereditary emphysema and cystic fibrosis in partnership with Bayer, a US pharmaceutical company. PPL had developed transgenic sheep that expressed the protein α_1 -antitrypsin in their milk, and was preparing to collaborate with Bayer to run large-scale human trials of the treatment.

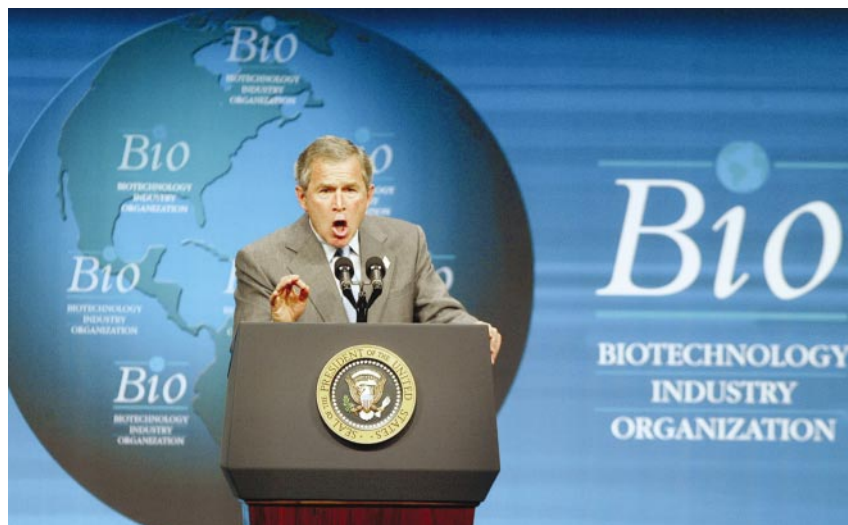
Bayer say they have now placed the trials on hold after PPL decided not to take on the £42 million of debt required to finance the building of a commercial-scale plant for purifying the protein from the milk.

"Without a production facility, you can do all the clinical trials in the world and there still won't be a drug at the end of it," says Tricia McKernan, who is director of communications for Bayer Biological Products at Research Triangle Park in North Carolina.

PPL now faces a difficult future. It has only one product that looks likely to become profitable — another protein called fibrin, a surgical 'glue' that can replace stitches and which is not derived using transgenic techniques. But the market for this is crowded, says Julie Simmonds, a biotech analyst at Evolution Beeson Gregory in London. "We remain unconvinced about the long-term outlook for PPL," she adds.

The business has already been heavily pruned. PPL sold its xenotransplantation arm this April, and closed down its stem-cell projects last September.

Geoff Cook, PPL's chief executive, says that the decision about what to do next lies with shareholders, and warns that they may decide that selling the company's remaining assets is the best way to recoup their investment. ■



Opening the coffers: George Bush espouses further federal investment in biotechnology.

Share slump brings biotech firms to government's door

Erika Check, Washington

The biotechnology industry met this week in the seat of the US government — Washington DC — and the location couldn't have been more fitting. Because while entrepreneurs claim to shun government as a slow-moving dinosaur, observers say it is currently biotech's best friend.

The renewed interest in government money comes during a worldwide economic downturn. Analysts say that the funding desert has made smaller biotechnology companies — usually wary of the obligations that come with government financing — highly dependent on federal resources.

"Biotech companies are saying that they can't get start-up funding, so what can fill the void?" says Stephen Davis, an attorney with Heller, Ehrman, White and McAuliffe in New York. "The only thing that can do it is the federal government."

The Biotechnology Industry Organization (BIO) engaged in an unusually extensive public-outreach campaign for its annual meeting, with a barrage of advertising in Washington newspapers and television channels, and a two-day festival on the National Mall. Carl Feldbaum, president of the BIO, says that the organization has staged large campaigns at its past three conventions. "But here in Washington we've extended that theme exponentially," he says.

Several factors have forced biotech firms to turn to the government, analysts say. Stock markets around the world have slumped, leaving companies unable to raise money through initial public offerings. Last year, only four biotech firms in the United States went public — a tenfold drop since 2000. In Europe,

only three biotechs went public last year.

Davis points out that early-stage biotech firms — those less than three years old, without a product ready to go to market — are having an especially hard time. Venture-capital funding for such firms totalled only \$65 million in the first quarter of this year — a 70% drop from the same period in 2002. "Venture capitalists used to fund preclinical work, but now they want things in late phase II clinical trials with good data," Davis says.

This has made federal agencies such as the National Institutes of Health (NIH) more popular. The NIH is mandated to spend 2.5% of its extramural budget — \$560 million this year — on small-business awards.

In a speech to the BIO convention on 23 June, President George Bush backed even more federal investment in biotechnology. He called on Congress to pass a piece of legislation — Project BioShield — that would give \$6 billion over the next ten years for research and development to counter bioterrorism.

As always in Washington, the money is flowing both ways. Republican legislators, in particular, have benefited from large contributions from biotech companies in recent years. In return, critics say, the legislators have worked to weaken the Food and Drug Administration, which regulates the industry.

But analysts say that an increasingly cosy relationship with the government won't save the biotech industry's skin. They point out that \$6 billion over ten years isn't that much, next to what private sources used to invest. "Venture-capital firms measure cash flows in the billions of dollars," says Joe Cortright, a consultant based in Portland, Oregon. "The government just cannot invest on the same scale." ■

Journals wrestle with definition of 'competing' interest

Jonathan Knight, San Francisco

Scientific journals have been striving of late for greater openness about the competing financial interests of their contributors. But a spat over a paper in *Science* suggests that clashing views over what makes a conflict of interest means full transparency is hard to achieve.

Earlier this year, Australian activists noticed that a 2002 paper on the spread of herbicide resistance from transgenic canola to nearby fields (M. A. Rieger, M. Lamond, C. Preston, S. B. Powles and R. T. Roush *Science* 296, 2386–2388; 2002) did not mention that two biotechnology firms — Monsanto and Aventis Crop Sciences (now owned by Bayer) — paid nearly 20% of the costs of the trials.

Alerted to the fact by a reporter for an Australian television programme in early May, *Science* contacted the authors for an explanation. *Science* requires contributors to declare financial ties that might be construed as influencing the outcome of their research.

The authors responded that they did not view the company funding as a conflict of interest. Industry co-sponsors don't participate in the design or conduct of the study, nor are they permitted to vet the findings or stop publication, claims co-author Christopher Preston, a molecular ecologist at the University of Adelaide. "I refuse to participate unless I can call the shots," Preston told *Nature*.

Although *Science* concluded that the funding did not amount to a conflict of interest, it has now revised its disclosure policy as a direct result of the incident, according to a statement provided to *Nature* on 23 June. Now, all funding sources must be revealed in the paper's reference section, *Science* says.

Many journals ask about conflicts of interest, but some authors don't realize that they have them, says Nicholas Cozzarelli, editor-in-chief of the *Proceedings of the National Academy of Sciences*. For instance, one contributor didn't feel that his position as chief scientist of a firm that was supporting his academic work needed to be mentioned as he didn't think the paper would affect the company's stock price, Cozzarelli says.

Nature has requested and published details of competing interests for every paper accepted since October 2001. Despite the rising number of researchers with ties to industry, of the 1,300 or so papers published under the policy, only 50 have declared competing interests. ■

India plans to bring neutrino science home

K. S. Jayaraman, New Delhi

Indian high-energy physicists are seeking to revive neutrino science in their country by building an underground physics laboratory. If all goes to plan, the proposed India-based Neutrino Observatory (INO) could be operational by 2010.

Atmospheric neutrinos were first detected in 1965 by a joint Indian–British–Japanese experiment at the Kolar Gold Field in southern India. But the facility was abandoned in 1992, and the mine that housed it closed.

Now, with Europe's main neutrino lab temporarily closed and plans for an American lab in trouble, India is spending US\$1 million on a feasibility study for setting up a facility of its own, using a huge slab of magnetized iron to detect neutrinos.

"The study will be completed by the middle of next year and then submitted for funding," says Naba K. Mondal, a particle physicist at the Tata Institute of Fundamental Research in Mumbai, and INO project leader. The project would cost a minimum of 2.5 billion rupees (US\$54 million). "We are proposing an Indian-based facility but are looking for international participation," Mondal says. The INO collaboration already has 55 members from 15 Indian institutions and universities.

Two potential sites for the lab have been identified — beneath the Nilgiri Hills in the southern state of Tamilnadu, and at Ramam in West Bengal. Both are man-made caves and have more than 1,500 metres of rock cover that would shield the laboratory from cosmic radiation.

Recent environmental problems at the Gran Sasso National Laboratory in Italy (see *Nature* **423**, 675; 2003) and delays over the US project (see *Nature* **423**, 674; 2003) "make the idea of a new laboratory in India more attractive to the particle-physics community", says Maury Goodman, a high-energy physicist at the Argonne National Laboratory in Illinois.

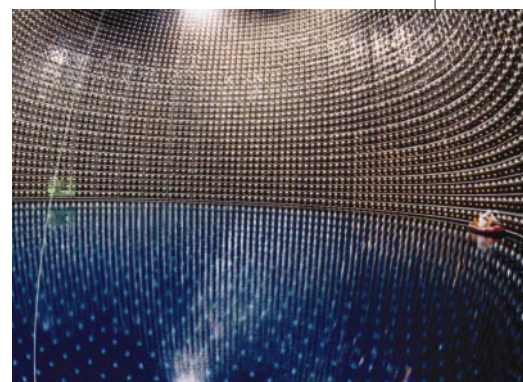
Physicists are keen to improve their understanding of the properties of the three different types of neutrinos — electron, muon and tau neutrinos. Mondal says that the new laboratory could verify results obtained by physicists at Japan's Super-Kamiokande detector suggesting that neutrinos can 'oscillate' between the three types. He claims that a few years of observations could confirm that neutrinos have mass and can oscillate.

This might be achieved by 'long-baseline' neutrino experiments to detect particles generated by distant accelerators in Japan, the United States or Europe. The extra distance would give neutrinos more time to oscillate between the source and the detector. "No other proposed facility has the

capabilities of this project," says Goodman.

John Learned, a neutrino physicist at the University of Hawaii, says that the plan "meets the criteria of being unique and taking advantage of local resources. It could achieve significant advances in particle physics and astrophysics."

But India may struggle to fund the facility. Final approval rests with the Department of Atomic Energy and the science ministry, whose budgets would have to support the neutrino observatory. Mondal says that funding will also be sought from the Ministry of Education, and adds: "If we undertake such a project, then we must have the involvement of Indian industry in a big way". ■



Shining example: Japan's Super-Kamiokande.

Discovery changes view of bacteria

Jonathan Knight, San Francisco

Bacteria have specialized compartments that were previously thought to exist only in higher organisms, according to research published earlier this month.

The discovery upsets a long-held notion about the nature of bacterial cells, researchers say. Whereas the cells of all other living things rely on a wide assortment of internal enclosures, known as organelles, to carry out their metabolic tasks, bacteria were thought to manage with a single open space devoid of partitions.

Now a team led by microbiologist Roberto Docampo at the University of Illinois in Urbana say they have found the first organelles in bacteria that have a direct counterpart in higher organisms (M. Seufferheld *et al.* *J. Biol. Chem.* doi:10.1074/jbc.M304548200; 2003). The organelles — called acidocalcisomes — are thought to store energy and help control acidity within the cell.

Docampo's bacterial organelles seem to be widespread; in fact, microscopists have been looking at them for a century without

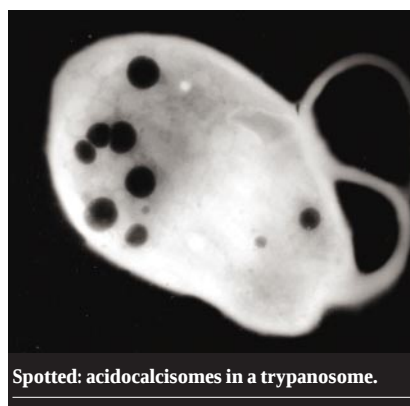
knowing it. They stain purple with certain dyes and have been called 'metachromatic' granules, but they were thought to have no membrane or enzymes — essential components of organelles.

Docampo has shown by electron microscopy and antibody techniques that metachromatic granules are surrounded by a membrane bearing proton-pumping enzymes that make its contents more acidic. For his experiments, he used *Agrobacterium tumefaciens*, a plant pathogen that causes crown gall. But a variety of other bacteria also have metachromatic granules, including the ulcer bug, *Helicobacter pylori*, and the bacterium that causes diphtheria, *Corynebacterium diphtheriae*.

The origin of the organelles is a mystery. In most cells, organelles fall into one of two categories. They are either components of an elaborate intracellular trafficking system, which bacteria lack, or they are derived from formerly free-living organisms that joined up with the ancient ancestors of modern cells.

Neither possibility seems particularly likely in this case, says Andrew Roger, who studies organelles at Dalhousie University in Nova Scotia and was surprised by the news. "Which of these might happen in bacteria is not clear," he says.

As one approach to this question, Docampo's group is now searching for more organisms with acidocalcisomes for comparison, by scouring sequence databases for the gene that encodes the proton pump. In addition, as the proton pump in the membrane is present in some harmful bacteria but not in human cells, drugs that target it might be effective against these infections, Docampo suggests. ■



Spotted: acidocalcisomes in a trypanosome.

KAMIOKA OBSERVATORY, ICRR, UNIV. TOKYO

COURTESY OF KILDARE MIRANDA

Whitehead geneticist quits for broad remit at medical institute

Washington Genome sequencer Eric Lander has decided to set up shop on his own. Lander is leaving his post as director of the Whitehead Institute at the Massachusetts Institute of Technology (MIT), a major player in the Human Genome Project, to start a research enterprise using a \$100-million gift from philanthropists Eli and Edythe Broad.

Plans for the Broad Institute were revealed on 19 June. Based in Cambridge, Massachusetts, it will have 12 primary and about 30 associated faculty members drawn from MIT, Harvard University and the Whitehead. Another \$200 million will be needed to run the institute, which will employ staff who work in biology, medicine, computer science, chemistry, mathematics and engineering.

The institute will be one of the more clinically oriented of a handful of new facilities that aim to do interdisciplinary work using the human genome sequence. "We're all trying to fulfil the promise of the new genomic medicine," says Lander. "But the Broad Institute will span biological and clinical research."



SOHO's antenna is again poised for action.

Repositioned Sun satellite beams in weather reports

London An important Sun-observation satellite survived a serious scare earlier this month when its main antenna stopped working. The Solar and Heliospheric Observatory (SOHO) has now been moved so that it can send signals to Earth, but it won't return fully to its former capabilities.

Scientists who use the joint US–European satellite for space weather forecasting feared that the data flow might halt when the antenna stopped moving properly. But the satellite has been reoriented so that its antenna points in the proper direction for all but two-and-a-half weeks of every three-month orbital cycle, says Bernhard Fleck, SOHO project scientist at the European

Space Agency. Most data lost during those periods can be recorded for later playback.

SOHO is now on its second mission, having finished its primary mission in 1998. Scientists hope that it can last until a successor, the Solar Dynamics Observatory, is launched in 2007.

Neutrino lab back on track after toxic leak

Munich Italy's Gran Sasso National Laboratory, an underground neutrino lab in central Italy, took a small step towards regaining normality last week.

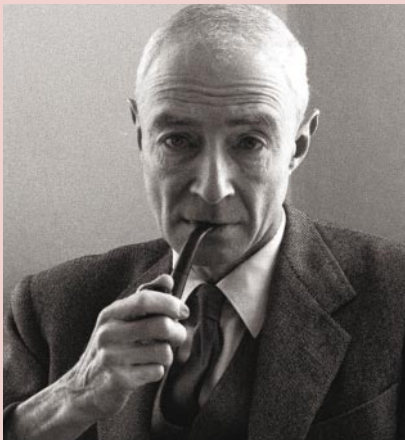
The installation of a new neutrino detector, which was halted in late May after toxic chemicals from the lab leaked into a nearby river, was allowed to resume by a local court. Work on OPERA, the Oscillation Project with Emulsion tRacking Apparatus, was given the all-clear on 17 June.

The detector, which will monitor a beam of neutrinos sent 730 km from CERN, the European particle-physics laboratory in Geneva, is being built in the same hall from which the toxic chemicals leaked (see *Nature* **423**, 675; 2003). The court's ruling affects only OPERA and some other smaller projects; most of the other work at Gran Sasso remains suspended pending an inquiry into the leak.

Father of atomic bomb gets night at the opera

San Francisco Celebrated US composer John Adams has been commissioned to write an opera based on the life of Robert Oppenheimer (pictured), the theoretical physicist who helped create the atomic bomb, and then went on to oppose the development of the more powerful hydrogen bomb. *Doctor Atomic* will premier in San Francisco in autumn 2005.

Meanwhile, the achievements of Albert Einstein are to be celebrated in a new ballet. Britain's Institute of Physics announced last week that it has commissioned the Rambert Dance Company to create the work, which will be premiered in London in May 2005. That year marks the centenary of the publication of three of Einstein's most important papers, including one on special relativity.



Researchers feel the power as electron laser lights up

Washington A device that may soon be classed as the world's most powerful free-electron laser has emitted its first light.

Researchers at the Thomas Jefferson National Accelerator Facility in Newport Beach, Virginia, turned on the device last week. Unlike conventional lasers, which use either semiconductors or gases to create

light, free-electron lasers use radiation emitted from electrons moving in a wiggly path (see *Nature* 415, 110–111; 2002).

The devices can easily be tuned by changing the frequency of the wiggling, and the US\$25-million machine at the Jefferson lab will be able to generate laser light across the infrared spectrum, with a maximum power of 10 kilowatts. An upgrade scheduled for about a year from now will allow the machine to

operate in the ultraviolet spectrum.

The laser will be used to study the structure of materials and individual molecules. The project has also attracted funding from the US Department of Defense, as the navy is interested in using free-electron lasers against anti-ship cruise missiles and other fast-moving targets.

Medical association backs human cloning

Washington After years of prevarication over the use of human cloning for stem-cell research, the American Medical Association has come out in favour of the technique. The announcement, made on 17 June at the association's annual meeting in Chicago, also said that physicians should be free to decline to participate in such research.

The association made it clear that it does not support the use of cloning for reproductive purposes. But it said that the cloning technique — known as somatic cell nuclear transfer — is likely to be valuable in treating human disease.

The US House of Representatives has voted twice to ban all forms of human cloning, but similar legislation remains stalled in the Senate. President George Bush has said he would sign such a ban should it be approved by Congress.

Back to 'plan A'

The received wisdom in AIDS vaccine research is that stimulating cellular immunity is more important than producing antibodies. But some experts are now reviving the antibody strategy, says Erika Check.

Few clinical-trial results have had as much riding on them as those announced in February by a small biotech firm called VaxGen. The company, based in Brisbane, California, was the first to push a vaccine against HIV into 'phase III' clinical trials — the final tests that help regulators decide whether a drug should be put on the market. If the vaccine had worked, it would have been a watershed in worldwide efforts to defeat a disease that kills some 3 million people each year.

But when the numbers came in, they told a disappointing story. VaxGen gave its product, called AIDSVAX, to 3,330 high-risk volunteers — mostly homosexual men — from North America and Europe. Of those people, 5.7% contracted HIV, compared with 5.8% of the 1,679 volunteers who did not receive the vaccine. Despite efforts by VaxGen to argue that AIDSVAX performed better in certain ethnic groups, it was clear that the vaccine was a bust.

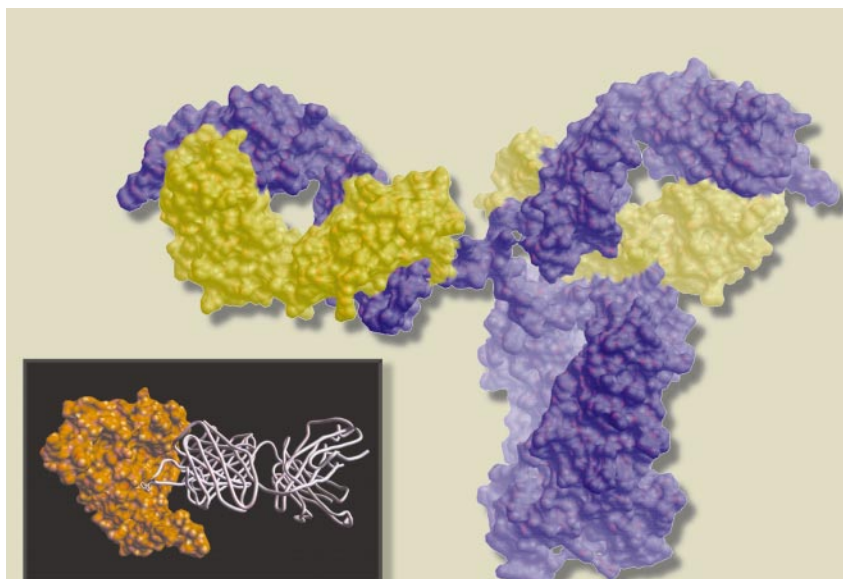
For many AIDS researchers, the vaccine's failure wasn't too surprising. VaxGen's charismatic co-founder, Don Francis, had raised the money to go ahead with phase III trials after the US National Institute of Allergy and Infectious Diseases (NIAID) decided not to bankroll the project. AIDSVAX consists of a protein called gp120, a major component of HIV's outer coat. It is designed to stimulate the production of antibodies that will prevent the virus from locking onto the immune cells that it infects, called helper T cells. Yet despite promising results from an early study in chimpanzees¹,

most of the human data on gp120 were extremely discouraging. In particular, antibodies taken from people immunized with the protein showed little ability in lab tests to prevent HIV from infecting its cellular victims².

Given these results, most AIDS vaccine researchers long ago gave up on the idea of stopping HIV in its tracks by stimulating the production of antibodies. Instead, they began to design a second generation of AIDS vaccines that would spur the body to make armies of killer T cells — the foot soldiers of our cellular immune response. Whereas antibodies can in theory lock onto viruses in the bloodstream and prevent them from infecting their target cells, killer T cells search out and destroy those host cells that have been infiltrated by a viral invader.

Many of these second-generation vaccines are now working their way through clinical trials. But for the most part, they show no signs of being able to halt HIV completely. "People are increasingly realizing that they're not going to be able to do

How do you design a vaccine that will elicit neutralizing antibodies instead of ineffectual molecules?



Pointing the way: the antibody b12 can effectively neutralize HIV. The secret to its success is its structure, which features extended 'fingers' (shown in purple, top right and left) that can reach and dock with a recessed pocket in HIV's frequently mutating protein coat (inset, shown in orange).

it all with cellular immunity," says Dennis Burton, an immunologist at the Scripps Research Institute in La Jolla, California.

So antibodies are coming back into fashion — but with a twist. Virologists now believe that AIDSVAX and other first-generation vaccines failed because they produced the wrong kinds of antibody. But certain super-antibodies may provide more powerful weapons in the battle against HIV. By marrying traditional immunology with techniques from structural biology, Burton and other researchers hope to cook up a third generation of AIDS vaccines that will selectively elicit the production of these 'neutralizing' antibodies.

Moving target

HIV is good at eluding most antibodies, thanks to a series of subtle tricks. First, the virus is sloppy at copying its genetic material, which causes frequent mutations that subtly change the sequence, and hence the shape, of its proteins. So as soon as the immune system assembles a library of antibodies that can lock onto the virus, the target proteins have changed sufficiently for the antibodies to struggle to maintain their grasp.

What's more, HIV has ways of fooling the immune system into making useless antibodies. For instance, when the virus prepares to enter a host cell, its surface proteins temporarily join together to form more complex structures. But because the immune system usually sees these proteins as singletons, it tends to make antibodies against them individually. The trouble is that

antibodies against the individual proteins cannot stop the virus from successfully infecting helper T cells.

Neutralizing antibodies exploit weaknesses in these viral defence mechanisms. One example is an antibody called b12, which recognizes gp120 and was isolated by Burton's group 11 years ago from a man who had HIV for six years, but had not developed AIDS. When they tested b12 in culture dishes, Burton and his colleagues found that it stopped most of the major strains of HIV from infecting human helper T cells³.

Perfect fit

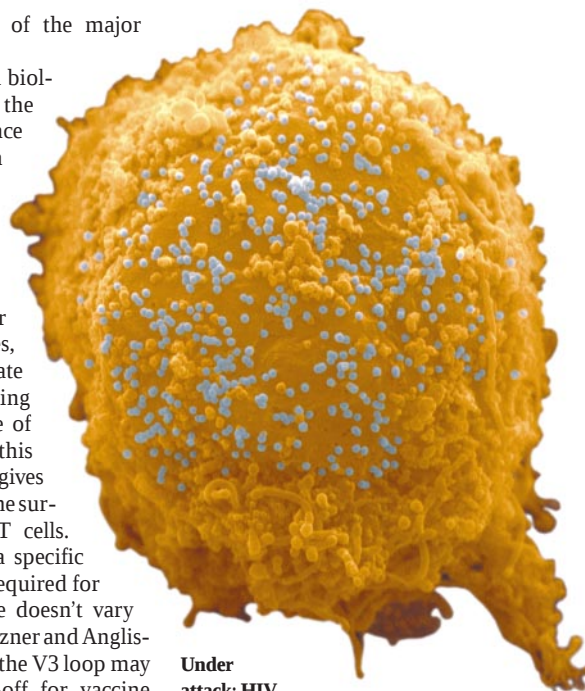
More recently, Burton and structural biologist Ian Wilson, also at Scripps, have taken a closer look at the structure of b12, and have found out why it is so effective: the antibody has a special finger-like protrusion that fits neatly into a fold in the gp120 protein⁴. This fold can't mutate very much, nor can it really change shape when the protein forms an infectious complex, because it needs to retain its ability to link up with a receptor on the surface of HIV's T-cell victims.

Another potent antibody against HIV — called 447-52D — also exploits the fact that certain parts of HIV's outer coat cannot change too much without compromising the virus's ability to infect cells. Like b12, 447-52D was found in a blood sample from a patient with HIV who had not developed AIDS. It docks to a part of gp120 called the third hypervariable region — or the V3 loop, for short. As its full name suggests, the V3 loop frequently mutates, which would seem to give antibodies against the region little chance of success. Yet

447-52D neutralizes most of the major strains of HIV⁵.

Working with structural biologist Jacob Anglister of the Weizmann Institute of Science in Rehovot, Israel, Susan Zolla-Pazner and her colleagues at New York University have revealed the secret of 447-52D's success. The structure of V3 mimics that of messenger proteins called chemokines, which help to regulate immune responses by binding to receptors on the surface of immune cells. Through this subterfuge, the V3 loop gives HIV another handhold on the surface of its target helper T cells. Again, 447-52D docks to a specific part of the V3 loop that is required for this binding, and therefore doesn't vary from virus to virus, Zolla-Pazner and Anglister found⁶. This means that the V3 loop may not, after all, be a write-off for vaccine development. "We've put it back on the map," Zolla-Pazner claims.

In work that has yet to be published, Burton and Wilson have analysed the structure of another broadly neutralizing antibody — called 2G12 — characterized in 1996 after being isolated from a patient by a team led by Hermann Katinger, now at the Austrian Institute of Applied Microbiology in Vienna⁷. At the Keystone symposium on HIV vaccines, held in Banff, Canada, in April, Burton revealed that 2G12 is actually made up of two antibodies joined together in a structure no one had ever seen before — which his group



Under attack: HIV (grey) infects and destroys helper T cells (orange), which are an important component in the body's immune response.

has dubbed a 'domain-exchanged dimer'.

This, says Burton, is an ingenious solution to one of HIV's notorious tricks. The virus cloaks itself in a coat of sugars that antibodies have a hard time distinguishing from those carried by the body's own cells. But the domain-exchanged dimer of 2G12 specifically recognizes the repeating pattern of sugars that is unique to HIV. "It's an ideal molecular solution to recognizing a tight cluster of repeating units," Burton says.

Reverse psychology

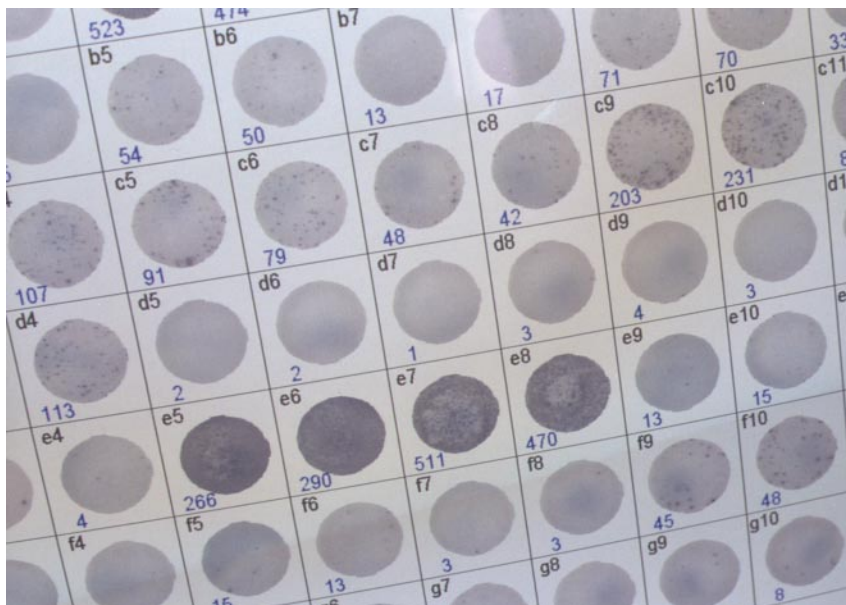
Understanding how neutralizing antibodies defeat HIV is important, but how do you design a vaccine that will specifically elicit their production instead of the usual panoply of ineffectual molecules? Burton calls the process "retrovaccinology", because it is working in the opposite direction to traditional approaches, which start with candidate vaccines and determine what antibodies they stimulate.

Retrovaccinology has never been tried before, and it won't be easy. But Burton is already thinking about how to provoke the selective production of 2G12. "We've dissected this antibody and shown exactly what it recognizes," he says. "So, if we immunize with a precise arrangement of sugars, we might be able to elicit an antibody like this."

Burton and Wilson have also engineered a gp120 protein that they hope will elicit the neutralizing antibody b12. The protein is capped by extra sugars in certain spots⁸. The idea is that these sugars will block the binding sites for the usual array of useless



VaxGen is continuing to test its AIDS vaccine in Thailand, although few expect it to prove successful.



Screen test: results showing which antibodies were produced by a patient given a trial AIDS vaccine.

antibodies elicited by the protein, causing it to stimulate the production of b12.

At the NIAID's Vaccine Research Center, which opened three years ago in Bethesda, Maryland, researchers are taking an approach that relies more on brute force. First, they induce mutations in HIV's coat proteins, then they analyse the structures of the mutated proteins to determine whether they are still likely to bind to known neutralizing antibodies. The idea is to produce a version of the proteins that won't induce the production of so many ineffectual antibodies, allowing the body's immune response to a vaccine to be dominated by neutralizing antibodies that could prevent infection. Gary Nabel, director of the Vaccine Research Center, says that this step-by-step approach has already yielded some good leads^{9,10}. "But we're not where we need to be yet," he notes.

Defensive formation

Given previous disappointments, few researchers believe that vaccines that induce neutralizing antibodies, by themselves, will provide complete protection against HIV. "To put up a brick wall against incoming viruses, the antibody has got to be there, and for those viruses that get through, you'll need cellular immunity," says Zolla-Pazner.

A bevy of vaccines aimed at stimulating cellular immunity are working their way through clinical trials. These use various methods to deliver genes from HIV into people, either on circular pieces of DNA called plasmids, or in gutted viruses that have been modified so that they don't cause disease. The viral DNA enters human cells, which translate it into proteins and display the proteins on their surfaces. The immune system then sees the proteins and — it is hoped —

responds by generating killer T cells primed to recognize and destroy any cells infected with HIV.

A diverse killer T-cell population has a better chance of recognizing the many possible forms of HIV. So a popular strategy for inducing cellular immunity these days is to use combinations of genes and delivery systems. Trials run by researchers at the University of Oxford, UK, working with colleagues in Kenya and Uganda, for example, are testing a vaccine that contains 20 different fragments of HIV genes. And a team based at Emory University in Atlanta, Georgia, has begun a trial in which a 'priming' shot of plasmid DNA will be



All change: Gary Nabel is creating mutant viral proteins in the hope of eliciting super-antibodies.

followed by a 'booster' package of genes delivered in a modified vaccinia Ankara virus (MVA).

Pharmaceutical firms and vaccine manufacturers are also getting in on the act. Merck in Whitehouse Station, New Jersey, and Aventis Pasteur in Lyon, France, have joined forces to test a different combination strategy. Volunteers first get a shot of Merck's vaccine, which contains HIV genes in a modified adenovirus. They then get Aventis Pasteur's booster, based on a bird virus that causes canarypox. Meanwhile, Wyeth, based in Madison, New Jersey, has invested in vaccines that deliver the HIV genes in the vesicular stomatitis virus, which causes disease in livestock but is harmless to people. Wyeth is also exploring ways to boost the power of its vaccines by delivering them with immune signalling proteins called cytokines.

Cold war

Immunologists are still trying to assess the quirks of these various approaches. Plasmid DNA stimulates only weak immune responses by itself, for example. And because adenoviruses cause common colds, many people might simply respond to Merck's vaccine by reactivating their previous anti-cold defences, rather than launching a new immune response that incorporates cellular immunity against HIV. MVA could run into the same problem. It was used in Germany to vaccinate against smallpox during the 1970s, and the US government is considering using it in this capacity to respond to potential bioterrorist attacks, because it is safer than the alternative vaccines.

"Every one of the cell-based strategies has a potential downside," concludes Norman Letvin, an immunologist and vaccine researcher at Beth Israel Deaconess Medical Center in Boston. "But we have a much clearer idea than we did five years ago of what we need to accomplish, and any one of the strategies being pursued may help us get over the top."

Ultimately, many vaccine developers believe that the final leg-up may come from some combination of vaccines that stimulates both cellular immunity and the production of neutralizing antibodies. But even the most optimistic aren't bold enough to say that success is a sure bet. "Whether we can really come up with the magic bullet is still unknown," says Nabel. ■

Erika Check is Nature's Washington biomedical correspondent.

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Hidden talents: JILA's unassuming exterior houses teams of skilled technicians whose support has helped to revolutionize atomic physics.

Rocky Mountain high

Beneath the peaks of Colorado nestles an unusual institute that leads the world in atomic physics. Peter Aldhous visits JILA, where a culture of sharing has underpinned Nobel success.

It's always pleasing to meet an award-winning scientist who is keen to share the credit with unsung heroes. Even so, Eric Cornell's response to questions about how he came to bag a share of the 2001 Nobel physics prize comes as a breath of fresh air. I might have expected him to credit an eminent professor who first set him on a scientific path that ultimately led to the creation of a new kind of matter. Instead, Cornell singles out his technicians. "We have a very high quality of support staff," he says.

After spending a day in Boulder, Colorado, as a guest of JILA, formerly known as the Joint Institute for Laboratory Astrophysics, it's clear that Cornell's comment is not simply the product of modesty. Each of JILA's staff repeats the mantra: the institute's enviable reputation stems in large part from its outstanding mechanical and electronics workshops, and their skilled and dedicated staff.

It's not the only distinctive theme. JILA is also a place where egos and empire builders are definitely not welcome. Dana Anderson, one of the lab's optical-physicists, notes that potential recruits must have a willingness to share resources and lab space to ensure that the most promising projects can flourish. "A fair amount of attention is given to whether

they'll be a good JILA citizen," he says.

World-class workshops, an open attitude to new ideas, plus stunning views of the Rocky Mountains — it sounds like a physics utopia. So how did this agreeable environment come into being? And in the cut-throat world of modern science, how does JILA's admirable ethos manage to survive?

Atomic adventures

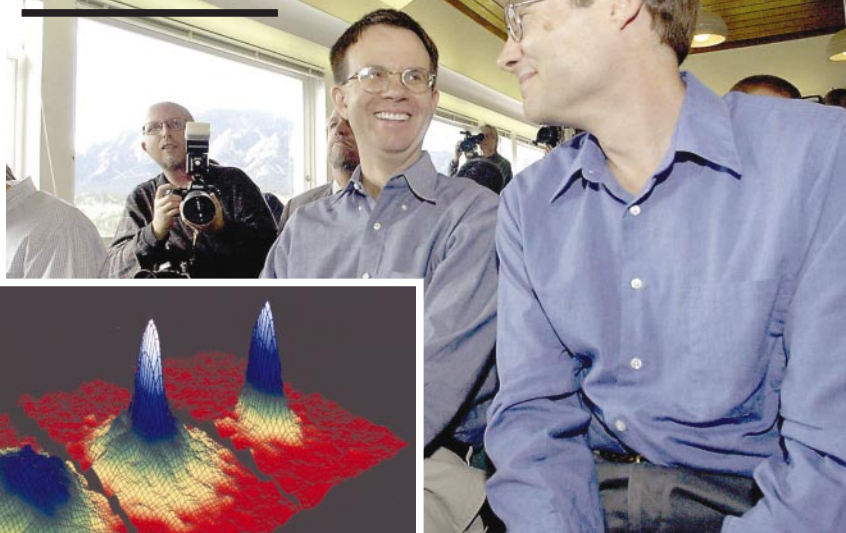
Before 1995, the lab was little known outside the fields in which it specializes: astrophysics and optics, together with atomic, chemical and materials physics, and precision measurements of phenomena, such as time and gravity. But in that year, Cornell and his colleague Carl Wieman put the lab firmly on the map of world science. By cooling a gas of rubidium atoms to within a whisker of absolute zero, they created a Bose–Einstein condensate — a state of matter whose existence had been theorized some 70 years before. This form is special because the rubidium atoms enter the same quantum-mechanical state as each other — the condensate, in effect, behaves as a single particle.

Six years later, Cornell, Wieman and Wolfgang Ketterle, whose team at the Massachusetts Institute of Technology created a

condensate shortly after the JILA group, were rewarded with a Nobel prize (see *Nature* **413**, 554; 2001). This honour has linked JILA's name forever with Bose–Einstein condensates, but Cornell and Wieman's achievements were in fact just one incarnation of the lab's adventures at the forefront of atomic physics, which date back more than four decades.

JILA was established in 1962 as a joint venture between the National Institute of Standards and Technology (NIST), a federal technology agency whose work includes the development of improved techniques for measurement, and the University of Colorado at Boulder. In those days, some of the hottest action in astrophysics lay in studying the atmospheres, or coronas, of stars such as our Sun. Understanding their dynamics required detailed observations of atomic collisions under extreme conditions, and so the marriage between atomic physics and astrophysics seemed perfectly natural.

But over time, JILA's astrophysicists moved onto other problems. So, too, did the atomic physicists. "The two groups got interested in different things," says Judah Levine, who works on super-accurate atomic clocks, and is currently JILA's chair. Today, this



Eric Cornell and Carl Wieman (right) won a Nobel Prize for creating a unique form of matter, the Bose–Einstein condensate (inset).

involves trapping them using lasers and magnetic fields. Thanks to JILA's technicians, Cornell and Wieman were able to conduct their experiments inside a bespoke glass vessel with the magnetic coils held outside. If Ketterle and his colleagues had a similar set-up, Cornell observes, they would not have experienced the delay of several weeks that they suffered when one of their coils melted, contaminating the entire apparatus.

Paul Beckingham, a PhD in astrophysics who heads JILA's electronics shop, says that building the specialized devices required by some of the institute's physicists requires some creative thinking. Finding the right electronic component, for example, can involve scouring obscure catalogues, such as those for vehicle parts. For some, this is an alluring prospect. "People who like this kind of life tend to stick at it," he says.

Speedy pursuits

The focus on creating the right environment for technically demanding experiments also extends to JILA's arrangements for purchasing supplies. "The time it takes from ordering a widget and the widget arriving is much shorter than elsewhere," says Cornell. "We put a lot of effort into it." And as someone who knows the value of being the first to achieve a significant experimental result, Cornell is in no doubt that this effort is worthwhile. "In research, anything worth doing is worth doing quickly," he grins.

But like any successful lab, JILA can't rest on its laurels. The reinvention of the lab's experimental programme is ongoing, with one important area being ultrashort laser pulses. Jun Ye, for example, is applying cutting-edge laser technology to a wide array of projects, including the development of improved atomic clocks, the fine control of chemical reactions, efforts to develop secure communications, and the imaging of living cells.

Still, the lab has suffered some setbacks, notably NIST's withdrawal of its support for JILA's astrophysics programme in the 1990s — although the pull-out happened over a sufficiently long period for it not to pose major difficulties. And although the University of Colorado has a respectable reputation, the fact that it isn't in the same academic league as the likes of Harvard and Stanford means that senior JILA scientists are always mildly anxious about their continued ability to attract the brightest postdocs and graduate students.

But all the signs are that there's no need to worry about recruitment. "We've done pretty well and are doing better," says Cornell, who notes that Boulder has some attractions that Harvard or Stanford could never match. "It's pretty nice here, if you're a climber, a skier or a cyclist."

Empire-builders, however, need not apply.

Peter Aldhous is Nature's chief news and features editor.

♦ <http://jilawww.colorado.edu>

amicable divorce is so complete that the phrase 'laboratory astrophysics' has no bearing on JILA's activities — hence the decision in 1995 to drop the full title in favour of the acronym. While JILA's astrophysicists are now exploring exotic phenomena such as supernovae and black holes, their former partners moved first into studying atomic properties using laser spectroscopy, followed by the work on ultracold atoms that led to Cornell and Wieman's prize.

The success of JILA's atomic physics programme is in part due to the security that stems from an unusual funding stream. For three decades, the lab has held a massive 'group grant' from the National Science Foundation (NSF), which today runs to \$3 million per year, about 10% of JILA's total operating budget. It's the way this money has been spent, says Wieman, that defines JILA's prevailing ethos.

Youth culture

Today, that grant is managed by Wieman, Cornell, and Carl Lineberger, a 35-year veteran of JILA who studies the structure and stability of ions. First, the principal investigators make sure that the workshops on which JILA's experimental expertise depends never have to scrimp and save — JILA spends some 10% of its budget on its workshops, about twice the sum put aside by most US academic physics departments. Second, the spending is under constant review to ensure that the most exciting projects win support. "We always give priority to new, younger people," says Wieman. "If they have new ideas, or they need some piece of equipment, we give it to them."

This sounds wonderfully altruistic, but Wieman stresses that the underlying dynamic is one of rigorous competition with the wider world of atomic physics. JILA's group grant must be renewed every five years, and this is no formality. "If they get

it renewed, it's because they damned well deserve it," says NSF programme director Barry Schneider. "They are excellent at making sure that they use this money only to fund the best of the best." Ultimately, argues Lineberger, it is the lab's ability to adapt to new scientific opportunities that has kept the grant going. "The reason it is in its 30th year is the fact that the science of the grant has reinvented itself several times," he says.

The group grant currently accounts for more than 12% of the NSF's total spending on experimental atomic, molecular, optical and plasma physics. "That's very conspicuous, and you have to be absolutely above any reasonable standard," says Wieman. As a result, the principal investigators are strict in cutting funding from any projects that prove less exciting than was hoped. "We're far more ruthless than other grants like this."

For those who make the grade, the environment fostered by the NSF group grant makes JILA something of a physics playground. "It has a different feel; senior people will give up space and money," enthuses Debbie Jin, whose team is currently striving to create another new form of matter similar to a Bose–Einstein condensate, but using atoms with different quantum-mechanical properties, known as fermions (see *Nature* **417**, 892–893; 2002). "The machine shop is outstanding, and the electronics shop is something you don't find in a physics department anywhere," she adds. "Experiments can go faster here."

Given this billing, I feel that I'm entering a revered inner sanctum when I finally visit JILA's workshops. They are, indeed, impressive — as is the range of skills possessed by the technicians who work within them. Cornell, for instance, partly attributes JILA's success in being first to create a Bose–Einstein condensate to the lab's in-house expertise in working with glass. Cooling the rubidium atoms

Dalí and the double helix

Science fascinated the great surrealist, who combined it with angels and allegories.

Sir — Some of the recent articles on the influence of DNA molecule structure and genetics on contemporary art (see, for example, www.nature.com/nature/DNA50) refer to Salvador Dalí, the renowned Catalan surrealist artist, and to one of his paintings in particular: *Butterfly Landscape, the Great Masturbator in Surrealist Landscape with DNA*.

Dalí (1904–1989) was highly interested in science. In the 1930s, his interest focused on dual images and illusions; in 1940 he turned to Planck's quantum theory; and in 1945 the nuclear, or atomic, period of his work began. In the 1950s, 'corpuscular' painting led Dalí to nuclear mysticism. Between 1955 and 1978, his work was deeply influenced by genetics in particular, and especially by DNA and its structure.

When Dalí read Watson and Crick's 1953 *Nature* article, he said: "It is the real proof of the existence of God." After that, DNA influenced his paintings and many other activities. DNA was present in at least nine paintings from 1956 to 1976: *Still Life, Fast Moving* (1956), "the decomposition of a fruit dish", metaphorically summarized man's post-atomic understanding of nature. Dalí suggested that there is still a cosmic order in the Universe by incorporating spirals into the composition; he felt that the spiral was the basic form of life, an idea that was confirmed by Crick and Watson in 1953.

Butterfly Landscape, the Great Masturbator in Surrealist Landscape with DNA (1957–1958) locates a prettified evocation of a space-filling model in one of Dalí's typically barren landscapes inhabited by sub-Freudian enigmas. The title of the painting *Galacidalacidesoxyribonucleicacid* (1963) combines DNA with Dalí's own name and that of his wife, Gala. It represents the three parts of existence: life (DNA structure), death (represented by men with rifles) and life after death (represented by God).

Dalí's fascination with the subject influenced a series of other paintings, some of them reworking similar themes and using similar titles. *Desoxyribonucleic Acid Arabs* was painted around 1963. *Hommage à Crick et Watson* (1963) — a name that was also given as a sub-title for the tongue-twisting *Galacidalacidesoxyribonucleicacid* — includes a picture of the scientists and the legends "Watson: a model builder" and "Crick: Life is a three-letter word". *DNA Representation (Jacob's Ladder)* (1971) was



A surrealist view of the meaning of life:
Galacidalacidesoxyribonucleicacid, 1963.

part of the tribute to F. Duran Reynals held at the 1971 National Conference of the Spanish Society of Biochemistry. The structure of DNA is mixed together with angels in Jacob's ladder. *Deoxyribonucleic Acid and Jacob's Ladder* (1975) is a

surrealistic representation of the DNA structure, with angels ascending and descending the ladder. Two paintings called *The Structure of DNA* (1975–1976), represent DNA structures with different coloured backgrounds.

Dalí was fascinated by DNA: "Every half of a shoot is exactly linked to its matching half, just as Gala was linked to me ... It all opens and closes and interlinks with amazing precision. Heredity depends on a sovereign mechanism, and life is the product of absolute rule of deoxyribonucleic acid".

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Crystal: ever-evolving and expanding in scope

Sir — In his Concepts essay "Crystal: In search of clarity" (*Nature* **423**, 485; 2003) Gautam R. Desiraju asserts that "to a scientist, 'crystal' denotes solid matter in a more or less ordered form ... According to the International Union of Crystallography, a crystal is any solid that gives a discrete X-ray diffraction diagram."

Yet there is more to crystals than solidity, for condensation into symmetrical arrays can be governed by repulsive as well as attractive forces. Some ordered arrays that fulfil the criterion of symmetrical diffraction consist not of atoms or molecules, but of discrete objects, or even organisms, such as the *Iridoviridae*. The rainbow diffraction of visible light by ordered arrays of hydrous silica microspheres is familiar as the play of colour in precious opal, but few realize that an equally arresting display can be seen when such microspheres are widely separated in aqueous suspension by electrostatic forces and Debye screening. The colourful diffraction testifies to long-range order, yet fish can swim through this crystalline glory and it reassembles in their wake.

There are also crystals of crystals, as monocrystalline nanoparticles are equally susceptible to falling into line in repulsive lattices, macrocrystallites of metals in planetary cores, and vast domains of crystalline order created within neutron stars by the strong nuclear force. Good luck in trying to detect their diffraction pattern.

At the limit of artifice are ordered arrays of atoms assembled by manipulation, which can defy the criterion of least energy that Desiraju's fascinating piece invokes. The many ramifications of this include the possibility of creating deliberate arrays of stable isotope atoms. This has important technical ramifications, for while the ballistic phonon free path, and the thermal conductivity of diamond (illustrated in the Concepts essay) can be enhanced by reducing the carbon 13 level in synthetic diamond feed stocks, that improvement can be transcended only when entropy as well as free energy is defied by ordering isotope defects instead of letting a random cloud of anharmonic oscillators destroy the thermal transport capacity of an otherwise perfect lattice (see R. Seitz, *Science* **250**, 1194–1195; 1990).

Desiraju is certainly right in observing that "the meaning and scope of the term 'crystal' can only evolve and expand".

Russell Seitz

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Crystal: clear difference from glass

Sir — In his otherwise excellent Concepts essay (*Nature* **423**, 485; 2003) Gautam R. Desiraju proposes that a scientist and a layperson may have different ideas about how to define a crystal. He even mentions that Spanish speakers "have only one word (*cristal*) for both 'glass' and 'crystal'."

This comment is not totally rigorous. The Spanish Real Academia dictionary (www.rae.es) shows two clearly distinct definitions that I have translated: "(1) glass (*vidrio*), (from Latin *vitrum*): solid, fragile material, transparent, without crystalline structure [...]; (2) crystal (*cristal*), (from Latin *crystallus*): solid material whose atoms and molecules are regularly and repeatedly distributed in space."

However, it is true that in Spain we use the word *cristal* for both concepts in colloquial terms.

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Gautam R. Desiraju replies — The word *cristal* is most commonly used in Spanish to describe crystal and glass, unlike the English language, where 'crystal' refers only to high-quality glass, cut glass and so on. The word *vidrio* is not used so frequently.

Online comment could promote unfair criticism

Sir — David M. Eagleman and Alex O. Holcombe in their Correspondence "Improving science through online commentary" (*Nature* **423**, 15; 2003) have proposed a web-based public arena for post-publication comment on scientific papers. Although we share these authors' concern that the slowness of peer-reviewed publication holds up comment and rapid (or even any) transmission of corrections, thereby impeding scientific progress, we believe that their proposed solution, a direct link between a website and PubMed entries, itself suffers from difficulties.

Perhaps the most worrisome issue is the likely prevalence of axe-grinding and hidden vested interests on a notice board that is meant for posting logical flaws, experimental weaknesses, questionable assumptions, alternative explanations and even non-replications. Although the proposed moderator could perhaps filter out overtly hostile or libellous notes, it is hard to imagine that he or she would be able to evaluate data adduced as evidence of non-replication.

The idea of post-hoc review is a good one, but Eagleman and Holcombe's proposed model amounts to a free-for-all, which might encourage ill-considered comment with the potential for damaging worthy science and scientists. One alternative is to provide independent scientific assessment of published papers directly linked to PubMed, as in a

subscription service in which we participate (see www.facultyof1000.com).

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MRC planning outweighs excellence in science

Sir — The comprehensive and harsh criticism of the UK Medical Research Council (MRC) by the House of Commons Select Committee for Science and Technology (see *Nature* **422**, 461 and **422**, 545; 2003) has been considered excessive by some but in my opinion is welcome: British science has found a friend. However, in view of the fact that the MRC supports about half the biomedical research in the United Kingdom, I am surprised that there has been no subsequent debate in *Nature* or the UK national press.

The select committee criticizes the MRC's practice of funding super-large research centres, long-running cooperative grants and interdisciplinary research groups, which leaves almost no funds for independent and young scientists. Closing or downsizing already existing centres with a reputation (such as the MRC's National Institute for Medical Research: see *Nature* **422**, 545 and **423**, 573; 2003) to free funds for a new one is even more puzzling.

I would like to give my own view on some of the MRC's problems. First, timing is crucial: creating a huge centre before the people who will run it are identified has risks. If the centre does not work, it is likely that its leader will retire before this becomes evident, and others will be blamed for the failure. In the meantime, other scientists will be deprived of funding.

George Radda, the MRC's current chief executive, says that if there had been no far-reaching goals 50 years ago, the double-helix structure of DNA would not have been solved. But this advance, as well as the solution of the first protein structures and protein sequencing, were made possible by three visionaries: John Bernal, Lawrence Bragg and John Randall. They had gathered the right people for the tasks and were powerful enough to obtain money from anywhere. The MRC deserves credit for funding these projects, but it did not hurry to create a new huge and expensive research centre in order to do so.

The MRC Laboratory of Molecular Biology (LMB) in Cambridge was created more than ten years later, after six Nobel prizes were awarded and the seventh was in the pipeline. At that time, the MRC recognized that there was already a critical mass of scientists with proven abilities and that funding such a large institution would repay the investment. They were right: the LMB kept attracting more able scientists and several more Nobel prizes were won subsequently.

My second concern is how and why the system of awarding the remaining scarce money for grants was changed. Peer-review assessment is fundamental to the funding of science. In the past, project proposals were assessed by three to four referees, chosen by the relevant MRC committee. Now, proposals are sent to 24 referees and judged when about eight have replied. It is hard to imagine that they would all be well-informed (in my field, there are not 24 centres anywhere that are actively involved in research). Thus, referees are chosen by administrators, who do not have a broad view on who is competent to judge a project, instead of being chosen by practising scientists. The power has shifted from scientists to administrators.

My third concern is the system of marking of the projects: alpha (recommended for funding), beta (with reservations) and gamma (failed). The alpha projects are additionally subdivided, the highest being alpha-A. Many projects are marked alpha-A, several times more than there is money to fund. They cannot all be equally good, but because they all have the same mark, the committee is given the power to choose which grants to support on political or 'strategic' grounds, independent of merit. Moreover, 'strategic' funding can prejudice the chances of other, perhaps better, projects, as the MRC will consider that it already supports work in that field and that it prefers to shift funds to another field in the strategic plan.

Dontcho Staynov

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Fiendishly clever?

Sir — Given the low regard in which lawyers are commonly held, was it just coincidence that led to your Special Report on scientists retraining for the legal profession appearing on page 666 (*Nature* **423**, 666–667; 2003)?

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Vision quest

A radical theory seeks to overturn current views of how we see the world.

Why We See What We Do: An Empirical Theory of Vision
by Dale Purves & R. Beau Lotto
*Sinauer Associates: 2002. 263 pp. \$42.95,
£27.99*

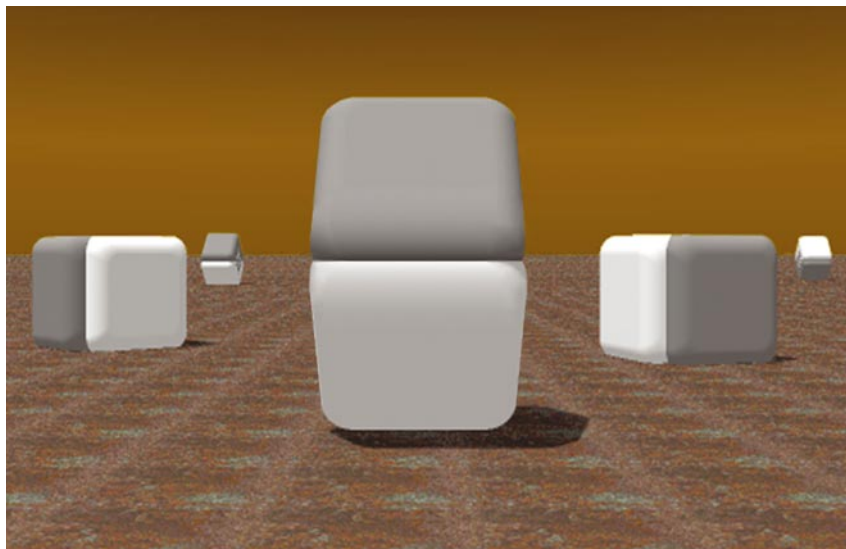
Michael Morgan

The standard model of vision, which this book ambitiously sets out to replace, begins with the retinal image being transduced into electrical signals by rods and cones. It is then analysed by banks of low-level sensors, such as retinal ganglion cells. To some extent these impose their own structures on the image, causing visual illusions such as Mach bands — the bright or dark regions perceived at the interface between bright and dark areas. The information transformed in this way is then used by higher-level mechanisms to infer the most likely structure of the outside world that caused the retinal images. This reconstruction can never be more than a best guess, however, because it is based on incomplete information from the two-dimensional image.

George Berkeley, in his *Essay Towards a New Theory of Vision* in 1709, thought the retinal image so unreliable that the true state of affairs had to be established by touch. The nineteenth-century physicist and physiologist Hermann von Helmholtz relented somewhat, and thought that the visual system could to some extent educate itself; he described vision as a process in which we infer the nature of the outside world from its images on the retina.

Fast forward to modern computational vision, as popularized by David Marr in his book *Vision* (W. H. Freeman, 1982), and we find that vision has been redefined. It is now described as an ill-posed mathematical problem (akin to deriving the number and speed of boats in a busy harbour from the ripples that they cause), but one that can generally be solved satisfactorily by making use of constraints derived from the most likely structure of the world.

Purves and Lotto will have none of it, except for the ambiguous nature of the retinal image, which is their starting proposition. Their 'empirical theory of vision' is that when we have a particular optical image on the retina, we experience a whole set of mental images with which it has been associated in the past, by either contiguity or similarity. We then have a perception that is some sort of combination of these images, either a mixture, a sum or a 'centroid' (it is not always clear which). At least, this is what I think they are saying. Perhaps the example of Mach bands will make the theory clear.



Seeing is believing: in the Cornsweet effect, the different brightness where the two central blocks meet creates the false impression that the rest of the lower block must be lighter than the upper block.

Mach bands arise at the extrema of linear luminance gradients, which we can think of as fuzzy shadows between bright and dark areas. On the bright side, observers see a thin white band; on the dark side they see a dark band. The Austrian physicist Ernst Mach deduced from this that some early filtering mechanism must be extracting the second derivative of the luminance profile, a guess later proved correct for retinal ganglion cells under appropriate conditions of illumination. Purves and Lotto show that these bands can also arise in real luminance profiles, such as those from the edge of a curved-edge cube lit from above and in shadow underneath. This is an interesting observation but doesn't explain why we see the bands when there are none in the image.

The crucial paragraph (and the pivotal section of the book) says, in part, this: "If the visual system evolved to see luminance relationships wholly on the basis of past experience ... then the perceptual response elicited by the Mach stimulus will necessarily incorporate into the ensuing percept the relative contribution of the sources to similar and even more distantly related stimuli in the past, including, in proportion to their frequency of occurrence, the highlights and lowlights in the luminance gradients associated with curved surfaces."

I read this as saying that some linear luminance gradients have real Mach bands, and therefore, that all linear luminance gradients have some Mach bands. Because they are all similar? Because they are associated in temporal contiguity? The answer is not clear

to me. What is sure, however, is that this is indeed a radical new theory. It has nothing to do with inferring the most likely nature of the outside object, as the bands are incidental features of illumination.

This impression is reinforced by analysis of the Chubb illusion, in which observers see a medium-contrast patch of texture apparently reduced in contrast when it is surrounded by texture with higher contrast. There is, as it happens, a plausible physiological explanation of this effect using lateral gain-control pathways in the visual cortex, but — as in the case of Mach bands, which are present in the output of retinal ganglion cells — Purves and Lotto have other ideas. The real explanation, they say, is that the image that evokes the Chubb illusion is similar to one that evokes the impression of translucence. They show that the Chubb illusion is abolished if the image is altered to make translucence a less likely interpretation. Our perception must therefore incorporate a little bit of translucence, making the low-contrast region of the image appear to have even lower contrast. This is the reverse of what we would expect from a reconstructionist theory such as that proposed by Helmholtz, according to which we would correct for the loss of transmission caused by translucence and see the patch more as it really is — as having higher contrast.

The sections of the book on brightness are the most interesting and challenging, with stunning illustrations and thought-provoking demonstrations. I found the other sections less compelling, particularly

the bit about geometrical illusions such as the Müller-Lyer and Poggendorf effects. For example, the authors ignore Glass's suggestion that the latter effect is enhanced by optical blur, along with virtually all other relevant psychophysical work. In general, the book is much stronger on polemic than on hypothesis testing.

After reading the book, I wasn't sure that I had understood the theory, so perhaps I have not done it justice. The summary at the end talks of retinal stimuli triggering 'reflex responses' that have been learned over time. There have been several behavioural theories of perception: is this another? Concerning reflexes, it is interesting to think of the celebrated McCullough effect, in which prolonged inspection of tilted, coloured lines causes achromatic lines of the same (but no other) tilt to have the complimentary hue to the 'conditioning' colour. According to the 'empirical theory', shouldn't they have the same colour? Shouldn't the 'waterfall after-effect' go in the same direction as the bayesian-biasing waterfall? Perhaps the theory can be refuted after all. ■

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Discovering geological time

The Man Who Found Time: James Hutton and the Discovery of the Earth's Antiquity

by Jack Repcheck

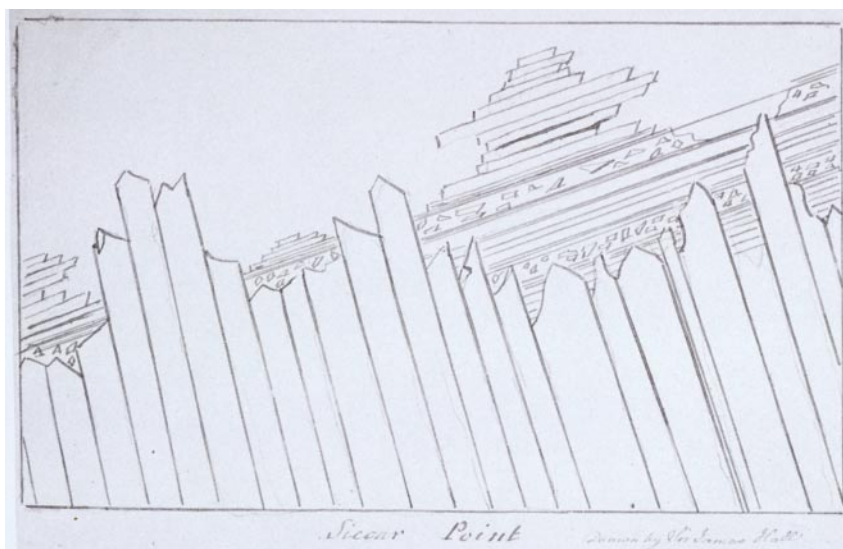
Perseus: 2003. 229 pp. US\$26, Can\$40

Simon & Schuster: 2003. £15.99

David R. Oldroyd

The Man Who Found Time is an example of a genre of science history that frequently appears in airport bookshops. The author takes an important figure or theme in the history of science — often one little known to the general public — and writes about it in a pleasant and engaging way, telling a story that deserves to be widely known. Such books seemingly make good money for publishers and authors, and are likely to be reviewed widely in journals such as *Nature*. So it is surprising how few professional historians of science have grasped such attractive opportunities.

These 'airport lounge' books seem to be creatures of publishers as much as authors. But why do publishers choose amateur science historians as writers? Who has the idea for these books: the authors or the publishers? Who does the real work in getting these books out? These thoughts are prompted by Jack Repcheck's enormous list of acknowledgements, among whom the agent, the



Rock solid: drawings of Siccar Point support James Hutton's ideas about the vastness of geological time.

publisher's staff and the editor are given special thanks. Repcheck also thanks various Hutton scholars for doing "research and spadework" without which the book "would have been impossible".

With all that back-up, one might expect a superior product. However, I was initially provided with a pre-publication version of the book. This gave me an insight into the author's uncertain grasp of the facts. The early copy was littered with so many errors that I asked to be supplied with the final version of the book before attempting a review. When it arrived, I was pleased to see that many of the obvious glitches had been dealt with (for example, Derby was now a town and not a river). But the principle of universal gravitation was still described as "the first natural law to be identified" (what about the laws of reflection and refraction?). Some erroneous definitions of geological terms had been corrected, but incongruities remained, such as the idea of "vacillation" between marine and terrestrial environments.

Having said this, the theme is worthwhile and the text is pleasantly written. After an (unnecessarily lengthy) account of how the idea of a young Earth was established, based on biblical and other historical records, Repcheck sketches some of the main events of Scottish eighteenth-century political history, Hutton's Edinburgh environment, his intellectual milieu, and his personality, career, ideas and achievements. He then gives an incomplete account of Hutton's scientific work, and carries the story through to the work of Charles Lyell and Darwin.

Naturally, I accept that Hutton was a pivotal figure in the history of geoscience and deserves to be much more widely known. He considered weathering and erosion, and developed a cyclic theory of Earth's history. Sediments were thought to be consolidated by the Earth's hypothesized internal heat.

From time to time, magma was supposedly intruded into the Earth's crust, elevating land and forming new rock that in time would weather and erode to form new soils and eventually sediments. So while constantly changing, the Earth is a grand system in which the formation and destruction of rocks is balanced and conditions suitable for human existence maintained.

Hutton predicted the occurrence of unconformities, and confirmed his predictions in the field. When he examined a remarkable unconformity on the Berwickshire coast (at Siccar Point) with friends, they felt they were looking into the "abyss of time". The immensity of time that Hutton's grand cycles of geological change required could be seen from the arrangement of the rocks. Without such work, Lyell and Darwin would not have been able to do what they did.

Repcheck outlines this argument satisfactorily, but there are serious gaps in his account. No mention is made of Hutton's work on philosophy or his unpublished treatise on agriculture (which adumbrated the idea of natural selection). Repcheck insists on the significance of Hutton's chemical ideas, but says nothing about his ideas on phlogiston and 'solar substance', which were important in his overall theory (to the extent that some Gaia aficionados regard him as one of their forebears). Little is said about Hutton's methodology, and there is nothing about his claimed intellectual debt to the earlier work of Robert Hooke (described in Ellen Drake's 1996 book *Restless Genius*), other than saying that Hooke's work offered one of the important precursory publications for Hutton.

More seriously, there is no mention of how Lyell's observations of the still-standing columns of an ancient building at Pozzuoli in the Bay of Naples influenced Lyell's ideas about elevation and subsidence. Even more

importantly, given the book's theme, nothing is said of Lyell's interpretation of the accumulation of Mount Etna's lava flows, which overlie geologically quite recent rocks, as evidence for the Earth's great age. But I should be surprised if the author knew about such matters, his knowledge of the history of geology literature evidently being limited.

All in all, the book reveals the author as an amateur historian of science, leaning on the work of others and not doing justice to his important theme. Yet the book will doubtless sell well with the publishers' backing. In contrast, another semi-popular, but authoritative, book by the Hutton scholar Donald McIntyre (with Alan McKirdy), *James Hutton: The Founder of Modern Geology* (The Stationery Office, 1997), received insufficient funding to accommodate references and had only a very brief bibliography and a modest print run. Why should this be so? ■
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Eyes on the prize

How to Win the Nobel Prize: An Unexpected Life in Science

by J. Michael Bishop

Harvard University Press: 2003. 320 pp.

\$27.95, £18.50, €27.95

István Hargittai

If offered reincarnation, the Nobel laureate J. Michael Bishop would choose to come back as a musician (with exceptional talent, to be sure), because he thinks that one lifetime as a scientist is enough. The son of a lutheran minister, he grew up in rural Pennsylvania, and became enchanted with research during his last years at Harvard Medical School. He has been at the University of California, San Francisco since 1968, and worked for 15 years with his former postdoctoral associate, and ultimately fellow Nobel laureate, Harold Varmus.

Their work had its roots in the discovery of a cancer-causing virus in chickens by Peyton Rous in 1911, who was awarded the Nobel Prize fully 55 years later. Five individuals then went on to win Nobel Prizes for related work. David Baltimore, Renato Dulbecco and Howard Temin won in 1975 "for their discoveries concerning the interaction between tumour viruses and the genetic material of the cell", and Varmus and Bishop became Nobel laureates in 1989 "for their discovery of the cellular origin of retroviral oncogenes". Baltimore and Temin found the viral enzyme reverse transcriptase, which allows RNA to be copied into DNA, a reversal of the normal flow of genetic information. This discovery could have been Bishop's had he been more daring. However,

Illustration

A fine body of work

Leonardo da Vinci's anatomical drawings, such as those of the shoulder shown here, may have been undertaken primarily out of an artistic desire to perfect his representation of the human body. They were influenced by the prevailing dogma of medical anatomy, as established by the Greek anatomist Galen some 1,400 years earlier. Leonardo's focus on drawing from life — in particular from his first-hand observation of human dissection — clearly informed his later works. The accuracy of his representations of human anatomy often surpassed that of contemporary medical experts. A massive book containing all of Leonardo's sketches, together with an analysis of the artist's work — *Leonardo da Vinci 1452–1519: The Complete Paintings and Drawings* (Taschen) by Frank Zöllner — has recently been published. *Mary Purton*



he learned his lesson and was fortunate enough to get another chance.

The discovery of oncogenes (cancer genes) raised the question of whether such genes might be present in the genetic composition of normal as well as cancerous cells. Locating them carried the promise of understanding human cancer at the genetic level. At first it was thought that oncogenes were viral genes, but Bishop and Varmus discovered that they were cellular genes that had been kidnapped by the virus. It took their team four years to identify them.

Bishop quotes a beautiful description of a moment of discovery by one of their post-docs, Dominique Stehelin: "The intensity of the emotion I experienced and the intellectual clarity induced by the situation at that moment were very special." Furthermore, "I suspect that few have the privilege of enjoying such a moment when one is intensely and profoundly aware that a major step forward in Science has been made, and that one has contributed to it." Alas, the quote was from an open letter to the Nobel Committee by Stehelin, who was not among the Nobel awardees. For every Nobel laureate there are others who might have also been included but were not, and every story about how to win the Nobel Prize may have its counterparts.

Bishop does not give a recipe for winning the prize, as any attempt to emulate a particular research career would be doomed to fail. However, throughout the book, he makes

important points that budding scientists may find useful. For example, it is more useful to learn from one's peers than from one's teachers. Start a research career in a place where you feel genuinely needed, rather than choosing somewhere for its prestige. Being a pioneer in research is fun, although it may bring more fame to be part of a team completing a discovery. Give a name to your discovery as soon as it is made. And finally, Bishop points out, good scientists should also market their ideas well.

Nobel laureates often seem to be standard-bearers for good causes, usually by signing petitions or making statements about issues with which they may not even be too familiar. Bishop's involvement in public causes has been different. He actively organized the participation of scientists in a non-partisan movement to increase legislative attention for science. Their high-level lobbying helped to achieve record support for research from taxpayer's money in the United States.

Bishop compiled his experience and ideas in this book for the general public. He also provides a crash course on the microbial world that is a gem of instruction without being condescending. And his copious use of art, including poetry, is a statement about the unity of the two cultures. ■

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The ascent of water

Melvin T. Tyree

The transport system that drives sap ascent from soil to leaves is extraordinary and controversial. Like their animal counterparts, large multicellular plants need to supply all their cells with fuel and water. For animals, the solution was the evolution of a vascular system, with a pump to circulate an isotonic blood plasma that prevented cell rupture through the osmotic inflow of water. Plants took a different route to solve the problem of osmoregulation, encasing each cell in a rigid exoskeleton, the cell wall. But this rigidity brought with it a lack of mobility — for whole organisms and also for tissues and cells. Plant tissues were too rigid to evolve a pump mechanism for long-distance transport. So what force is responsible for the ascent of water in plants?

More than a century ago, H. H. Dixon (1896) proposed that a pulling force was generated at the evaporative surface of leaves and that this force was transmitted downward through water columns under tension to lift water much like a rope under tension can lift a weight. The cohesion–tension theory (C–T theory), as it is known, supposes both adhesion of water to conduit walls and cohesion of water molecules to each other.

Francis Darwin, when commenting on Dixon's proposed theory, said: "To believe that columns of water should hang in the tracheals like solid bodies, and should, like them, transmit downwards the pull exerted on them at their upper ends by the transpiring leaves, is to some of us equivalent to believing in ropes of sand."

Dixon proposed that plants transport nearly pure water in the xylem conduits — the woody channels that run from soil to leaves — at negative fluid pressures. Plants seem to retain and transport water in conduits while under pressures as negative as -1 to -10 megapascals (MPa) — that is, pressures 10 to 100 times more negative relative to atmospheric pressure than a perfect vacuum. I can think of no other botanical theory that has engendered more incredulity among physical scientists and animal physiologists than the C–T theory, because it requires us to suppose that water is transported in a metastable state. If an air-bubble or vapour-void of sufficient diameter were to arise in a xylem conduit under negative pressure, the water column would cavitate and the void would expand to displace the water, making the conduit dysfunctional. Direct measurements of negative pressure in xylem made with a cell pressure-probe 5–15 years ago failed to confirm the C–T theory. But an improved pressure-probe technique has now proved that the mechanism functions as supposed. How do plants do it, and what other limitations on plant performance result?

Negative pressure is generated by surface tension (capillarity) that arises at the air–water interfaces (menisci) at the cell-wall surfaces of leaves, where a system of pores about 20 nm in diameter can, theoretically, sustain negative pressures down to about -15 MPa before a meniscus is sucked through the cell wall to seed embolisms in adjacent xylem conduits. Water in conduits under negative pressure is metastable and hence should cavitate. The propensity of metastable water to cavitate is indeed the principle argument that many have used to reject the C–T theory. But cavitations frequently occur, and it is this exception that proves the rule. Cavitation events can be detected acoustically, and the overall impact of cavitations can be measured by loss of hydraulic conductance, which in different species can be reduced to half at negative pressures from -0.5 to -9 MPa. Cavitations are confined to single conduits. The C–T mechanism works in spite of the high probability of millions of cavitations in conduits, because there are billions to trillions of conduits in a tree and because adjacent conduits are isolated from each other by primary cell

Plant hydraulics

When you're a large organism and made of wood, you can't have a heart or other contractile organs, but you still need to move fluids to live. How is this done?

walls in pits. Conduits are interconnected by multiple adjacent conduits, providing redundancy in multiple pathways for water movement should one conduit cavitate. The pore diameter in primary cell walls determines how negative the pressure can be in a water-filled conduit before cavitations are seeded from an adjacent, embolized conduit.

Using a large number of small-diameter conduits increases redundancy and stability in the transport of metastable water — but there are trade-offs. The Hagen–Poiseuille law tells us that the hydraulic conductivity of a conduit should be proportional to the fourth power of the lumen diameter. Hence, as conduit diameter drops, the pressure difference from root to leaf required to maintain an adequate water flow rate increases, and this sets a practical limit of minimum conduit diameter between 5 and 10 μm . At the other extreme, the upper limit of conduit diameter obtained by natural selection seems to be about 500 μm . There seems to be a trade-off between large, efficient conduits and increased vulnerability to cavitation in plants. There is also growing evidence that the hydraulic conductivity of plants limits the maximum rate of gas exchange and carbon gain, so the typical conduit diameter of a species can limit the maximum height the species can reach. Fast-growing species have large, efficient conduits that are highly vulnerable to embolism; such plants perform poorly in drought. Slow-growing species have small, inefficient conduits that are very resistant to cavitation.

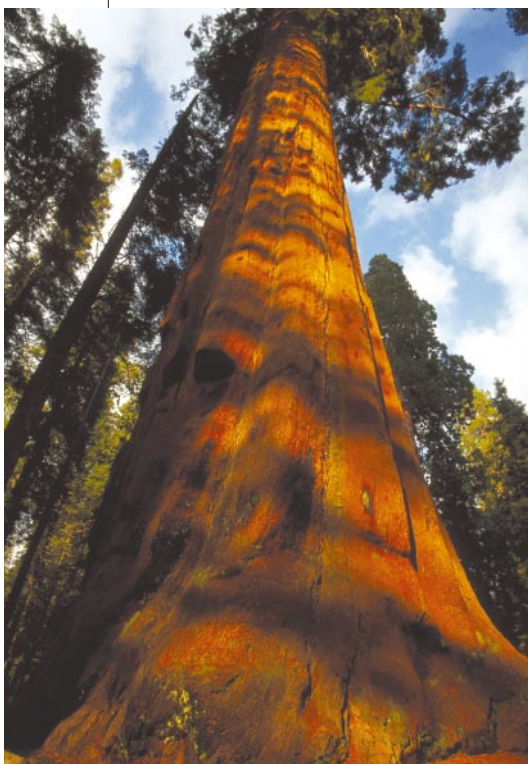
Other trade-offs that have driven the evolution of diversity in land plants were unexpected — trees with dense wood are strong but hydraulically inefficient. An understanding of this legacy of natural selection should allow us to breed or engineer improved drought-resistant or fast-growing trees. ■

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FURTHER READING

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P. SCHERMEISTER/CORBIS



Trees transport metastable water by a mechanism unparalleled in engineering science.

Synthetic sea shell

Michael Rubner

The mechanical properties of natural substances such as bone and shell are envied by those involved in the fabrication of materials. A 'bricks-and-mortar' structure, assembled layer by layer, is the key to making sea shells.

For a materials scientist, cross-sectional images of the complex microstructures of naturally occurring hard materials such as bones and sea shells are awe-inspiring. Over many millions of years, nature has devised schemes to combine seemingly incompatible building-blocks — 'soft' organic proteins and 'hard' inorganic particles of calcium carbonate — in a manner that produces composite materials with the unusual combination of high strength, hardness and toughness. Imagine, however, that you could build such a structure as a mason would, one layer at a time, from the bottom up. Writing in *Nature Materials*, Tang and colleagues¹ explain how it can be done, using a molecular-level processing scheme known as layer-by-layer assembly^{2,3}.

Flexible soft materials that can undergo energy-absorbing molecular rearrangements during deformation are tough, but also very compliant. In contrast, rigid hard materials are stiff but often also very brittle, and they have little ability to absorb energy, so their toughness is low. To be strong, hard and tough, a material must be able to absorb a large amount of energy during mechanical deformation and also maintain high stiffness. In bone or shell, this desirable combination of properties is made possible by one key attribute — a bricks-and-mortar-like structure, made up of strongly interacting, nanometre-size building-blocks. The 'hard' bricks and 'soft' mortar are complementary in their response to stress and strain.

So far, attempts to mimic these structures with synthetic building-blocks have failed to produce a material with similarly impressive mechanical properties, because most conventional processing techniques simply do not offer the nanoscale level of control needed to create a highly regular bricks-and-mortar-type arrangement. Nature has no such difficulty with nanoengineering: it can assemble, in a regular manner, building-blocks of the right dimensions that interact strongly enough at their interfaces to allow the transfer of deformation energy between the rigid bricks and the softer mortar. Reproducing these elements synthetically is a challenge.

But this is exactly what Tang *et al.*¹ have achieved, through the alternating sequential deposition of negatively charged, nanometre-thick clay platelets (the bricks) and a positively charged polymer (the mortar).

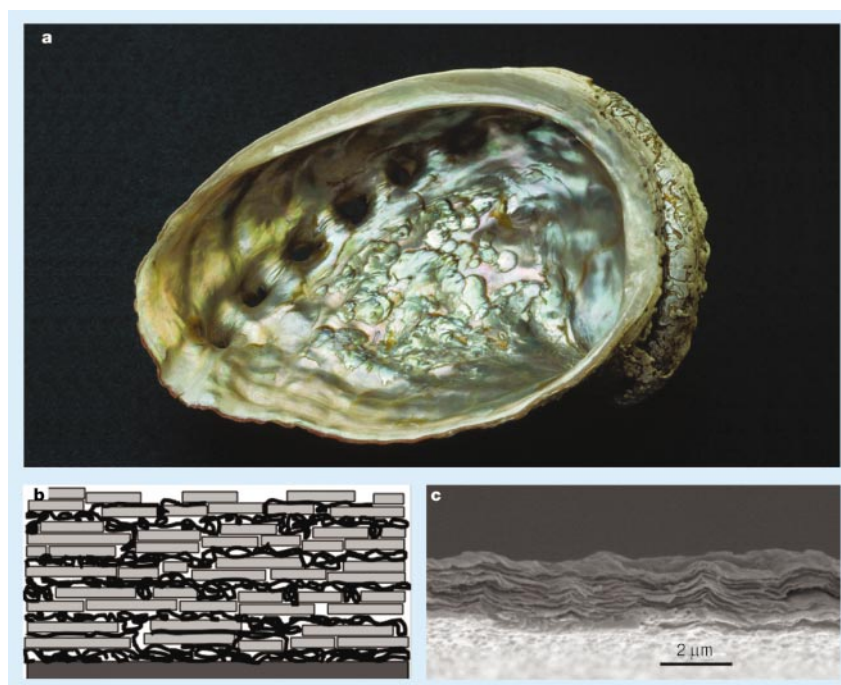


Figure 1 The 'bricks-and-mortar' approach. **a**, The natural strength, hardness and toughness of bone and shell are attributable to their nanoscale structure of calcium carbonate bricks and mortar-like protein layers. By mimicking this structure, Tang *et al.*¹ have created a new material with mechanical properties similar to nacre, or mother-of-pearl. **b**, Montmorillonite bricks (0.9 nm thick) are deposited layer by layer above a silicon-wafer substrate, alternating with polymer chains of mortar. **c**, Structures that are many layers deep can be built — this one has 100 brick and polymer layers — although the process is slow.

The primary driving force for this adsorption-based assembly, which is carried out entirely from dilute aqueous solutions of the materials, is electrostatic: the positively charged polymer chains are attracted to the negatively charged clay platelets, and vice versa. By assembling the clay platelets (in this case, a material called montmorillonite) and polymer chains one deposition step at a time, the authors are able to create a bricks-and-mortar-type arrangement that mimics the natural structure of nacre, the material known as mother-of-pearl (Fig. 1).

The polymer chains are arranged in coils and folds, physically pinned by relatively weak electrostatic interactions. As the material is deformed and the clay platelets begin to slide over each other, the polymer chains can undergo molecular rearrangements, through the breaking of 'sacrificial' ionic bonds and the concomitant unfolding of the coiled polymer chains. And this process, in turn, allows the material to absorb a lot of

deformation energy. The authors report that thin films assembled in this way have tensile strength and stiffness that approach those of seashell nacre. Their stiffness is significantly higher than the more disordered composites previously fabricated from similar materials using conventional methods.

As a step towards creating synthetic materials that truly mimic the mechanical behaviour of naturally occurring materials, this is an important advance. The true potential of this approach — to construct more complicated layered structures containing many types of building-blocks — has yet to be pursued. But, for example, this group has also shown that layer-by-layer assembled films containing carbon nanotubes have exceptional mechanical properties⁴. So this seems a promising way of fabricating multilayered, multi-component thin films, with molecular architectures designed to take full advantage of the complex interactions possible between many different types of materials. And as this

J. A. SUGAR/CORBIS

assembly process simply involves alternately dipping a substrate into dilute aqueous solutions of oppositely charged materials, it is easy to control the number, type and sequence of layers added to the film⁵.

There is a price to pay, however, for assembling the building-blocks one layer at a time. Such structures, particularly if they need to reach thicknesses in the micrometre range, require far longer fabrication times than more conventional processes such as spin-coating. Indeed, it is impressive that Tang *et al.*¹ have succeeded in creating free-standing, micrometre-thick films in this way (Fig. 1c).

Another challenge in working with charged polymers is that they are capable of adsorbing a lot of water, which in turn can degrade the mechanical properties by screening the ionic interactions that lend strength to the material. Tang *et al.* show that there is a significant decrease in mechanical performance when films are tested in high-humidity environments. But this problem

can be addressed, as many different types of materials can be assembled in these multilayer films, including those that are hydrophobic (water resistant) or that can be rendered hydrophobic by subsequent chemical or thermal treatments.

In any event, these model structures are sure to provide new insight into the behaviour of naturally occurring materials. Their applications could be widespread, from synthetic engineering of biological hard tissue to thin-film protective coatings.

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Telomeres

Taking the measure

Vicki Lundblad

Telomeres — the tips of chromosomes — need to be preserved, and this involves replenishing telomeric DNA when it has been eroded. But telomeres must not become too long, and one aspect of length control is now revealed.

In every organism, maintaining the integrity of the genome is a crucial endeavour. One aspect of genome maintenance involves protecting telomeres, the natural ends of linear chromosomes. This task is achieved by a suite of specialized protein complexes, which are anchored to chromosome ends through their association with further proteins that bind directly to telomeric DNA. The resulting structure prevents events that would be catastrophic for the genome, such as the loss of terminal DNA sequences or end-to-end chromosome fusions.

One of the complexes involved in telomere maintenance is an enzyme called telomerase, which adds DNA back to telomeres that have become eroded. Several other proteins also regulate this complex. But how the different proteins talk to one another — to keep telomeres the right length, to protect them, and to replicate them during cell division — is poorly understood. Writing on page 1013 of this issue and in *Current Biology*, respectively, Loayza and de Lange¹ and Colgin *et al.*² describe a crucial feature of the process by which telomerase can sense, and thus regulate, the length of individual chromosome ends.

Telomeric DNA is composed of G-rich repeats — reiterations of a short DNA sequence that does not code for protein and

is high in guanine (G) nucleic-acid bases. It also has a single-stranded stretch that overhangs the end of the double-stranded (duplex) telomeric region. This overhang is

the substrate for telomerase, which elongates chromosome ends by adding G-rich repeats. The importance of telomerase is evident from studies of yeast and human cells in which reductions in telomerase levels produce a steady decline in telomere length that eventually blocks cell division. Not surprisingly, then, telomerase is highly active in systems such as the blood and reproductive system, which rely on continuous replenishment through cell proliferation³. Much to the interest of cancer biologists, telomerase levels are also increased in most human tumours, providing a potential target for the development of anticancer drugs⁴.

In normal cells, telomerase activity is carefully controlled by several mechanisms. For instance, subunits that are part of the telomerase complex itself can positively regulate the enzyme, for example by mediating recruitment of the complex to chromosome ends⁵. Surprisingly, proteins that bind to the duplex region of the telomere can also be potent regulators, even though they do not appear to associate physically with telomerase. These duplex-binding proteins — which include Rap1 in budding yeast and the TRF1 and TRF2 proteins in human cells — can 'count' the number of G-rich repeats and, when telomeres become overly long, inhibit further telomerase activity^{6,7}.

Missing from this elegant proposal for telomere-length regulation, however, is an explanation for how information from the duplex portion of the telomere is relayed to the very tip of the chromosome — the site of telomerase action. To address this, Loayza and de Lange¹ and Colgin *et al.*² turned to a recently discovered human protein called

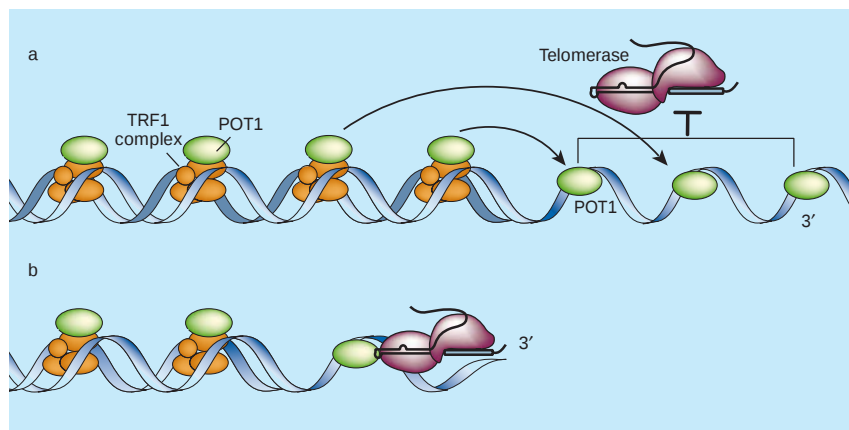


Figure 1 Model for telomere length control. Telomeres are found at the end of linear chromosomes, and their length must be precisely regulated — a process that involves the POT1 protein in human cells^{1,2}. Loayza and de Lange¹ have shown that POT1 binds to the TRF1 complex on the double-stranded (duplex) portion of telomeres. TRF1 complexes sense the length of the telomere, and the authors propose that this information is transmitted to telomerase (an enzyme that extends telomeres) via POT1, by transferring POT1 to the single-stranded overhang at the telomere tip. **a**, When the telomere is long enough, the levels of POT1 on the overhang are high, and telomerase is inhibited. **b**, When the telomere is too short, little or no POT1 is transferred to the end, and telomerase is no longer inhibited, allowing it to add DNA back to the telomere. Colgin *et al.*² have proposed that POT1 may also act as a positive regulator of telomerase when present at the single-stranded terminus. It might do so via a direct interaction with telomerase, in an analogous way to how the yeast Cdc13 protein regulates telomerase^{5,11}.

POT1 (ref. 8). POT1 is related to well-known proteins that bind single-stranded telomeric DNA in ciliates and budding yeast^{9,10}, and it itself binds to single-stranded telomeric substrates *in vitro*⁸. Loayza and de Lange show that, consistent with this biochemical property, POT1 is present at the tips of human chromosomes, and that this association is reduced when the length of the G-rich single-stranded overhang is decreased. But POT1 does not rely solely on its nucleic-acid-binding properties to bring it to telomeres. It is also found in a complex with the duplex-binding protein TRF1 (along with other TRF1-associated proteins, collectively referred to as the TRF1 complex). So, POT1 interacts with two different sites on telomeres: the extreme terminus, and along the duplex region.

What happens if POT1 loses its ability to interact with one of these two sites? Loayza and de Lange tested this by removing the DNA-binding portion of the protein, creating a derivative called POT1(Δ OB). This derivative still associated with the TRF1 complex, and hence with duplex telomeric DNA. But it could not interact with the single-stranded telomeric ends, and this resulted in a profound disruption of telomere length, with telomeres becoming rapidly and extensively elongated. These observations establish that POT1, like TRF1, is a negative regulator of telomere length. More significantly, because POT1 can interact with both the site of telomerase action and the duplex portion of the chromosome, it could be the missing link in the telomere-repeat-counting model.

Loayza and de Lange therefore propose that information about telomere length, which is measured by TRF1 through its ability to bind duplex telomeric repeats, is transferred to the tip of the telomere through the interaction between POT1 and TRF1. This interaction presumably affects the amount of POT1 that is loaded onto single-strand overhangs: the more telomeric repeats there are, the more POT1 is transferred to the telomere tip, and the greater is the reduction in telomerase activity (Fig. 1). The next question is how POT1 relays information to telomerase. It might act as a negative regulator by directly binding to the enzyme complex and inhibiting its catalytic activity. Alternatively, through its DNA-binding activity, POT1 might sequester telomeric DNA and thereby block access of telomerase to its substrate.

The picture is also far from complete in other respects. For instance, POT1 might not function solely as a negative regulator: Colgin *et al.*² propose that it also positively controls telomerase-mediated telomere elongation. The idea that a protein that binds single-stranded telomeric DNA can serve as both a positive and a negative regulator of telomere length has also been established in studies of the yeast Cdc13 protein^{3,11}.

Meanwhile, TRF2, like TRF1, binds duplex telomeric DNA and similarly contributes to the feedback mechanism that monitors telomere length⁷ — yet it does not interact with POT1 (ref. 1). This implies that TRF2 must have a different partner that transmits its signal to the chromosome terminus.

A separate question about POT1 centres on its postulated role in protecting chromosome ends (as opposed to maintaining telomere length) in human cells. Fission yeast lacking POT1 have a severe defect in end protection⁸ — will the same be true in humans? These open questions suggest that the intense focus on telomere biology is unlikely to abate anytime soon. ■

Atmospheric physics

Electric jets

Victor P. Pasko

Powerful electric currents have been detected in discharges between thunderclouds and the upper atmosphere. Carried by gigantic jets, they are a new factor in the model of the Earth's electrical and chemical environment.

Although cloud-to-ground lightning is a familiar disruption in the modern electronic world, lightning formed above the clouds is also an important factor in what is known as the global circuit of atmospheric electricity. Radio atmospheric emissions are natural electromagnetic emissions from lightning discharges and can propagate thousands of kilometres through the 'waveguide' formed by the Earth's surface and the ionized region of the upper atmosphere, known as the ionosphere. In 1925, the physicist C. T. R. Wilson wrote¹: "The discharges above the cloud would doubtless give rise to atmospheric electricity. If, as has been maintained, atmospheric electricity frequently originates in regions of rain unaccompanied by thunder, they may in such cases be due to discharges of this nature."

Wilson was right: on page 974 of this issue², Su and colleagues report their observations of several large-scale discharges, branching upwards to an altitude of about 90 km from the top of thunderclouds in the South China Sea; no associated lightning discharges were detected in the underlying thunderstorm. Radio atmospheric emissions recorded at remote locations in Japan and Antarctica, however, showed that there was a significant flow of current that moved several tens of coulombs of negative charge upwards from the thundercloud to the lower ionosphere.

Although eyewitness reports of events such as these — known as 'transient luminous events', or TLEs — have been recorded for more than a century, the first image of one was captured³ only in 1989, serendipitously during a test of a low-light television camera. Since then, several different types of

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TLEs above thunderclouds have been documented and classified^{4–6} (Fig. 1, overleaf). They seem to be fairly common in most regions of the globe, appearing over North, Central and South America, Europe, Australia, Japan and China. TLEs are known to be associated with large volumes of ionization, creating electrical paths through the atmosphere⁷ and causing significant perturbations of long-range communication signals⁸. But the effects of this ionization and its associated currents on the Earth's electrical and chemical environment are not fully understood.

In a simplified picture of the global electrical circuit, the Earth's surface and the conducting atmosphere above it can be imagined as plates of a giant spherical capacitor, with a potential difference of about 300,000 volts between them⁹. There are many components contributing to the balance of potential between the plates, but two are critical: thunderstorms, of which there are about 2,000 globally at any given time and which act as batteries charging the capacitor; and fair-weather regions, in which the capacitor can discharge continuously through the weakly conducting atmosphere, with a global leakage current of about 1 kiloampere.

The upper plate of the capacitor is not confined to a single level, but rather is distributed through the atmosphere, reflecting changes in atmospheric conductivity and the fair-weather electric field (so that the density of the leakage current in fair-weather regions remains roughly constant with altitude). In fact, most of the 300,000-volt potential drop between the capacitor plates happens within



100 YEARS AGO

The cleanliness of electric lighting has always been urged as one of the great claims in its favour, and it has been justly pointed out that the saving effected in redecoration partly balances its extra cost. Although this is true, electric light cannot be regarded as perfectly clean; it has long been noticed that there is a marked tendency for dust to accumulate on electric light fittings and wires, and on the walls and ceilings in their immediate neighbourhood. This is partly, no doubt, due to the air currents produced by the local heating, but it is also partly an electrical phenomenon. The dust particles floating in the air are presumably at air potential, and are consequently attracted to the conductors on the non-earthed side of an earthed system; they either stick to these permanently, or remain on them until charged, when they are projected on to and stick to the walls... If switches are always put, as they should be, in the non-earthed wire, the deposition of dust will only occur during the time the lamps are alight, and will be minimised. Mr. D. S. Munro, writing in the *Electrical Review*, points out that a still further improvement can be effected by using concentric flexible conductors instead of the ordinary twisted cord, the outer conductor being connected to the earthed side of the system.

From *Nature* 25 June 1903.

50 YEARS AGO

During the course of an ecological investigation of the polychaete annelid *Pygospio elegans* Clap., a remarkable mode of asexual reproduction was noticed... The bodies of the adults, both males and females, divide into pieces consisting of varying numbers of segments, generally about three or four, sometimes up to seven; several times fragments consisting of one segment only have been observed. Fission takes place in any part of the body. When just separated, the single fragment looks as if it had been cut off with a knife... Every single fragment is able to form a new individual exclusively from its own tissue. The rate of regeneration is very high, and at 20 °C. the regeneration of a new animal was completed in eight days; then the asexually formed individual will start a new division process.

From *Nature* 27 June 1953.

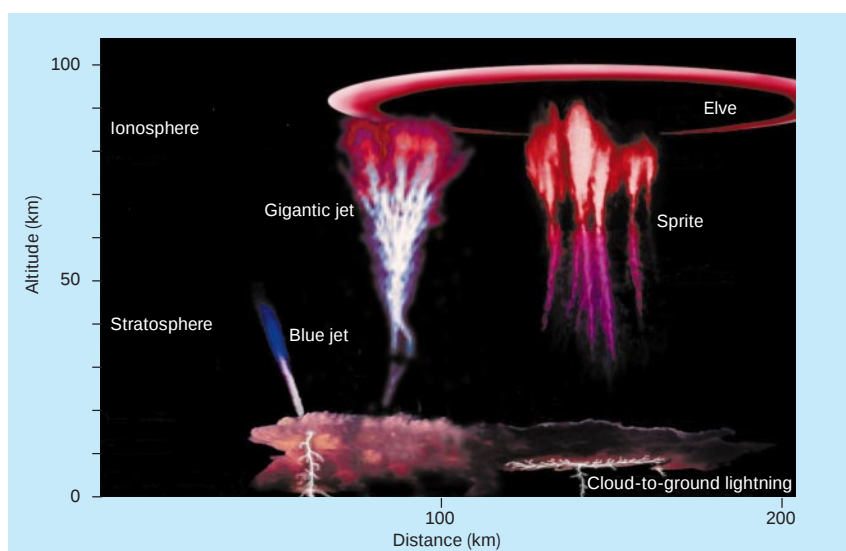


Figure 1 Lightning-related transient luminous events (TLEs). Several types of TLEs are known, and some examples are shown here: relatively slow-moving fountains of blue light, known as 'blue jets', that emanate from the top of thunderclouds up to an altitude of about 40 km; 'sprites' that develop at the base of the ionosphere and move rapidly downwards at speeds of up to $10,000 \text{ km s}^{-1}$; and 'elves', which are lightning-induced flashes that can spread over 300 km laterally. Su and colleagues² now report their observations of several gigantic jets, which propagated upwards from thunderclouds to altitudes of about 90 km. The strong emission of electromagnetic radiation from these events, detected as radio atmospherics thousands of kilometres away, indicates that several tens of coulombs of negative charge were transferred from the thundercloud to the lower ionosphere. (Graphic adapted from ref. 15, with the permission of the American Geophysical Union.)

a few tens of kilometres of the Earth's surface. For instance, 'blue jets' — TLEs that terminate at altitudes of around 40 km (Fig. 1) — probably move some charge to the upper plate of the capacitor. But no associated radio atmospherics have been detected for these events, probably because they take much longer to develop than the more impulsive jets reported by Su and colleagues.

Atmospherics of the strength recorded by Su *et al.* have previously been observed only in conjunction with the most powerful cloud-to-ground lightning discharges and the TLEs triggered by them, known as 'sprites'¹⁰. The authors admit that there may be a slight chance that the atmospherics they detected were associated with cloud-to-ground lightning discharges in the underlying thunderstorm — but then these discharges must have been repeatedly missed by the local lightning detection network, which is unlikely. However, it is clear that the events observed by Su *et al.* are very different from sprites, which typically start at altitudes of about 70 km and propagate downwards: the gigantic jets seen by Su and colleagues branch upwards from thunderclouds, spreading to a diameter of about 40 km at an altitude of 85–90 km (Fig. 1).

The ionization created by a gigantic jet is likely to have a significant chemical effect on that volume of atmosphere. In fact, the occurrence and dynamics of many TLEs, including those observed by Su *et al.*, closely resemble the behaviour of 'streamers' —

miniature needle-shaped filaments of ionization, commonly observed when an electric field is applied to a small volume of relatively un-ionized ambient air at ground pressure. Streamer discharges can lead to significant power losses on high-voltage transmission lines and can damage insulating materials; a streamer plasma of hot electrons embedded in cooler air is a good source of highly reactive species for use in the chemical treatment of hazardous and toxic pollutants¹¹. Because streamer filaments have high electric fields around their tips, streamer plasmas can easily generate electrons with sufficient energies to dissociate atmospheric oxygen molecules. The dissociation initiates a chain of reactions that leads to the formation of ozone in air (this process has been used for industrial ozone production for more than a century¹¹).

As atmospheric pressure is much lower at ionospheric altitudes than at the Earth's surface, streamers that would have diameters of a fraction of a millimetre at ground level instead appear as channels of glowing plasma that are many kilometres long and a hundred metres in diameter — easily observable above thunderclouds by low-light imaging systems deployed hundreds of kilometres away¹². High-altitude streamers also have the ability to produce highly active chemical species and can effectively 'treat' thousands of cubic kilometres of atmosphere. The branching observed in atmospheric TLE discharges, including those documented by Su and

colleagues, is also known in ground-level streamers (and is recognized as an important parameter for effective chemical treatment of large gas volumes¹³). So the known chemical impact of streamers may be a good indication that TLEs noticeably affect the chemistry of the atmosphere.

This field is in its infancy, and it remains to be seen how important the electrical and chemical effects of the gigantic jets and other TLEs are for our planet. The large quantities of negative charge transported by these jets, discharging the atmospheric capacitor, may have a strong influence on the voltages and currents in the global electric circuit. The events seen by Su *et al.*² seem to be a property of oceanic thunderstorms, and a global survey of these and other types of TLEs is planned, using Earth-orbiting sensors^{6,14}. Knowing how frequently these events occur will help us to understand their contribution to the global electrical circuit. ■

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Evolution

The battle between the sexes

Tom Tregenza

Male–female conflict over mating rate can drive rapid evolution and lead to female refusal to mate with males from other populations, so implicating sexual conflict in the generation of biodiversity.

Sexual conflict is a pervasive feature of the living world. Although the sexes need one another, they rarely have exactly the same priorities. Males can often increase their reproductive success — the number of offspring they sire — simply by mating with as many females as possible. Females, on the other hand, are limited by their ability to produce offspring, and unnecessary matings may be costly. This difference sets the stage for an evolutionary arms race in which males are continually evolving new adaptations to get females to mate with them rather than with other males, and females are striving to resist this manipulation.

On page 979 of this issue¹, Martin and Hosken provide evidence that sexual conflict can indeed drive very rapid evolution of female willingness to mate and of male traits that promote matings. They show that this process can lead to females being less ready to mate with males from other populations. This type of reduction in matings between populations could eventually lead to a complete lack of interbreeding, at which point the two groups would have become separate species.

Martin and Hosken took the dung fly *Seppis cynipsea*, males of which can be seen harassing females on cowpats throughout Europe, and set up three types of laboratory populations. Monogamous populations had

females kept with only one male, eliminating conflicts of interest altogether. ‘Conflict’ populations had either 50 or 500 flies in the same-sized plastic box, with equal numbers of males and females. Flies kept at higher densities mated more frequently, and the females lay fewer eggs, presumably because they are continually having to fend off amorous males. After two-and-a-half years and 35 generations, females were tested for their willingness to mate, both with males from their own population and with males from an independent population of the same type. As predicted, females from monogamous populations (having been under no selection for avoiding males) were the most willing to mate. Females from conflict populations were not only generally less willing to mate, they were also even more reluctant to mate with males from a different population than they were with males from their own population.

There were also differences between the two types of conflict population. At the larger population size, females evolved even greater discrimination against males from populations other than their own. This is particularly interesting because it is exactly the opposite of what is predicted by theories of speciation that are based on the idea that smaller populations diverge rapidly. When populations are small, the frequency of different genes can change very rapidly — each

type of gene is found only in a few individuals and so the prevalence of particular genes is strongly affected by chance events that happen to their carriers. The faster progress towards speciation in the larger populations seen in Martin and Hosken’s study is, however, exactly what is predicted by speciation theories based on selection that include sexual conflict². In large populations, selection will be more effective at finding genes in females that can counteract manipulative genes in males, because there will be more genetic variation available and because selection is not swamped by chance events.

But there is an alternative explanation for the pattern seen. The important thing might not be the fact that some populations were larger, but that they were at higher density, and hence experienced greater levels of conflict. This will have increased the pressure on females to be more reluctant to mate, which could have similar effects — evolution is faster because selection is stronger, while in the previous case evolution is faster because there is more variability for it to work with.

The study of the evolutionary role of sexual conflict is still in its infancy and, as might be expected, Martin and Hosken’s study raises as many questions as it answers. In particular, the finding that females are more resistant to males from populations other than their own runs counter to several studies³ that have found females with lower resistance to foreign males. These previous results have been taken as support for the idea that sexual conflict is characterized by males continually evolving new ways to manipulate females, who in turn evolve new methods of resistance⁴ — that is, females are poor at resisting male tactics they have not evolved with. Perhaps the explanation for Martin and Hosken’s different results is that by artificially increasing the level of conflict, they have created a situation where, instead of following male adaptations, females are leading the evolutionary dance, evolving new criteria that males must meet in order to be granted a mating. Hence, males that have been able to adapt to the preferences of females from their own population are at an advantage over foreign males.

This view has similarities to the type of argument used in models that examine the potential for differences in female mate preferences to drive speciation⁵, and highlights a second issue. Although there is clearly a lot of sexual conflict in Martin and Hosken’s system, it is possible that there is also sexual selection of the more familiar type in which females are choosing the ‘best’ males. The laboratory is very different from the wild, and females might simply be picking males better at dealing with this new environment, with chance differences between populations in the male traits that females use for mate choice.

This study shows that simply changing

the size and density of populations provokes rapid evolution in mating traits. A powerful approach to examining the factors that may be important in determining whether conflict creates diversity will be to conduct similar experiments using a range of animals and independently changing factors such as the number of individuals and the environment they experience. We also need to start examining the nature of the signals that males use to persuade females to mate, and how these are received by females. Combining these

approaches will provide new insights into how conflict can create diversity.

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Catalysis

A greener solution

Walter Leitner

A catalyst dissolved in a liquid phase can be recycled if the reaction products are captured in an adjacent supercritical phase. A catalyst-storing liquid that is plentiful and environmentally friendly has been found.

Catalysis is the key to sustainable synthetic chemistry. Homogeneous catalysts, dispersed in a solution of reactants, have many potential advantages over solid-phase, heterogeneous ones: the molecular nature of the catalyst means that it can be designed rationally to be selective and highly active. The difficulty is in separating out the products and recycling the catalytic material. An elegant solution to this problem, however, is ‘multiphase catalysis’¹: the catalyst resides in one liquid phase and the reactants and products are dissolved in a second phase. Supercritical carbon dioxide² — carbon dioxide under pressure, in a phase neither truly liquid nor gas — has been found to be a useful reactant/product phase in such systems^{3,4}. In the *Journal of the American Chemical Society*, Heldebrandt and Jessop⁵ introduce an attractive system using poly(ethylene glycol), a material that is benign and readily available, to immobilize a homogeneous catalyst while an adjacent phase of supercritical CO₂ (scCO₂) ensures selective extraction of the product.

Classical multiphase catalysis relies on two immiscible liquids, one dissolving the catalyst and the other containing reactants and products¹. Water is often used as the catalyst phase in combination with organic solvents such as diethyl ether, benzene or methylene chloride. Although this approach is generally referred to as ‘biphasic catalysis’, a reaction system that involves gaseous products will in fact have three phases (two liquids and the gas). In contrast, the system of liquid and supercritical phase, as used by Heldebrandt and Jessop⁵, is truly biphasic (Fig. 1), as any gaseous reagents are miscible in the supercritical medium. Using scCO₂ also eliminates any toxicological or ecological hazards that might be associated with the use of organic solvents.

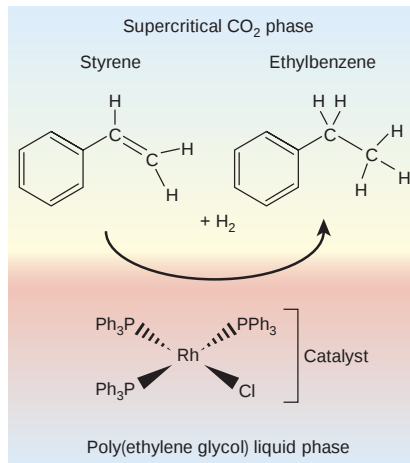


Figure 1 Biphasic catalysis. Heldebrandt and Jessop⁵ have devised a catalytic system comprising two phases, one liquid and the other a supercritical fluid. The reactants for the hydrogenation of styrene are dissolved in supercritical carbon dioxide; a liquid phase of poly(ethylene glycol) holds the catalyst for the reaction, which is a rhodium compound known as Wilkinson's complex. The two phases are immiscible, but mass transport between them is sufficient to aid the catalysed production of ethylbenzene. 'Biphasic catalysis' is not new, but this demonstration of poly(ethylene glycol) as a recyclable liquid catalyst phase could be a step towards 'green' catalysis on an industrial scale.

The choice of the liquid phase that will hold the catalyst is crucial. It should have a minimum solubility in scCO₂, but still allow efficient mass transfer with the reactant/product phase to catalyse the desired reaction effectively. Water and ionic liquids have been used successfully, but both combinations have their limitations^{3,4}. In the water–scCO₂ system, all reaction compo-

nents must be stable against acidic conditions (carbonic acid solutions have a pH of around 3) and many catalysts must be modified to be sufficiently soluble in the water phase. Ionic liquids are molten salts consisting of large, cationic organic molecules and suitable anions that are liquid in the typical temperature range of organic reactions (0–100 °C). Held together by electrostatic forces, typical ionic liquids are insoluble in scCO₂. But carbon dioxide does have a very high affinity for ionic liquids, which ensures rapid mass transport between the two phases⁶. For practical applications, however, high material costs and the current lack of toxicological data are drawbacks — although rapid progress on these points can be expected^{7,8}.

The Jessop group has been actively involved in developing biphasic catalysis with both water–scCO₂ and ionic-liquid–scCO₂ combinations^{9,10}. Consequently, the new approach⁵ is largely complementary to these systems. Heldebrandt and Jessop's choice of liquid catalyst phase is poly(ethylene glycol), or PEG. This material is widely used in food and beverages and for medical purposes, which is a testament to its benign character. At the same time, liquid PEG can dissolve many organometallic compounds that would typically be used as homogeneous catalysts without any need for their structure to be modified.

Under ambient conditions, only PEG with a fairly low relative molecular mass forms liquid phases — but then its solubility in scCO₂ is so high that the combination is useless for biphasic catalysis. Heavier PEG materials are waxy solids, but pressurizing them with CO₂ lowers their melting points enough to render the PEG phase liquid at reasonable reaction temperatures. (For example, PEG with an average molecular mass of 1,500 has a melting point of 48–51 °C under ambient conditions, but it can be used as a liquid catalyst phase at 40 °C under CO₂ pressures higher than 90 bar.)

Heldebrandt and Jessop⁵ used a rhodium compound known as Wilkinson's complex — (Ph₃P)₃RhCl — as a prototypical homogeneous catalyst to demonstrate the feasibility of their approach. This versatile compound can be used to catalyse a wide range of chemical transformations. In this study, the hydrogenation of styrene to ethylbenzene was the chosen test reaction (Fig. 1). Although this reaction is not synthetically important (styrene is in fact produced by the reverse reaction), it is often used to benchmark new multiphase catalyst systems. The authors show that, on extraction of the ethylbenzene product from the scCO₂ phase, the catalyst remained stable in the PEG environment and could be recycled four times without noticeable loss of activity. Rhodium contamination in the product was below the detection limit (less

than one part per million). Using PEG with an average molecular mass of 900 as the liquid catalyst phase, measurable amounts of PEG were detected among the extracted products; but for PEG with a mass of 1,500, the contamination was found to be as low as 0.1% by weight.

Heldebrandt and Jessop's approach to homogeneous catalysis is an attractive addition to the concept of multiphase catalysis with scCO_2 . The PEG catalyst phase is non-toxic and readily available, and, as has been demonstrated for ionic-liquid- scCO_2 systems, liquid- scCO_2 catalysis can operate efficiently under continuous-flow conditions^{11,12}. Engineering for this system would be largely identical to that of heterogeneous catalysis with supercritical fluids, for which a commercial-scale plant has just been opened in Britain¹³. Even though important data — such as long-term stability, reaction rates and leaching under continuous-flow conditions — have yet to

be collected, the PEG- scCO_2 system has clear potential for practical application in 'green' catalytic chemistry.

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Neurobiology

Synapses unplugged

J. Troy Littleton and Morgan Sheng

At the junctions between two neurons, the machinery that releases neurotransmitter from one cell must lie near calcium channels and align with detectors in the receiving cell. We now have a better idea how this occurs.

The computational power of the brain depends on the precise connections, or synapses, that link together the many billions of nerve cells. On page 939 of this issue, Missler and colleagues¹ describe their studies of how the neuexin family of proteins contributes to synapses *in vivo*. These proteins were believed to help induce synapse formation between neurons in culture dishes — but, as is so often the case, matters have proved rather different in live animals.

Specialized to allow rapid (millisecond) neuronal communication, synapses work broadly as follows. In response to an electrical impulse, the terminal of a 'presynaptic' neuron releases chemical neurotransmitters, which diffuse across the synaptic cleft to activate specific receptors on the postsynaptic neuron. These receptors cause an electrical discharge in the postsynaptic cell, thereby propagating the electrical signal.

More specifically, since the classic work of Bernard Katz², it has been known that electrical impulses propagating down a neuron cause an influx of calcium ions through voltage-gated calcium channels in the presynaptic nerve terminal; this in turn triggers neurotransmitter release. The rapidity with which calcium influx leads to neurotransmitter release (within 200 microseconds) means that the voltage-gated calcium channels must be very close to — perhaps even

physically associated with — the molecular machinery that releases the neurotransmitter from the cell^{3,4}. Furthermore, the presynaptic and postsynaptic elements of the synapse must be aligned, such that neurotransmitter release occurs at sites precisely

opposite clusters of neurotransmitter receptors in the postsynaptic membrane. An attractive idea is that this alignment is achieved by adhesion molecules — specific cell-surface proteins located on both sides of the synapse that grip each other across the synaptic cleft and hold the presynaptic and postsynaptic apparatuses in register.

One family of cell-surface proteins implicated in synapse formation and adhesion is the neuexins. Originally identified by their binding to α -latrotoxin⁵ (a spider toxin that triggers neurotransmitter release), the neuexins are presynaptic transmembrane proteins that have a large extracellular region and a short intracellular tail. A striking feature of neuexins is their molecular diversity: they are encoded by three genes, each of which has two regulatory regions; this means that long (α) and short (β) neuexin proteins can be generated from each gene. In addition, the messenger RNAs encoded by each gene can be processed in different ways (by 'alternative splicing'), resulting in thousands of distinct protein forms⁶.

Because of their great diversity and their presence on the surface of presynaptic terminals, neuexins became attractive candidates for determining the specificity of synaptic connections⁶. Consistent with this idea, specific neuexin proteins bind through their extracellular domain to neuroligins⁷, a family of transmembrane receptors found in the postsynaptic membrane, and to dystroglycan⁸, a cell-surface protein of unknown function in the brain. The interaction of neuroligin and neuexin in cultured neurons induces morphological events resembling synapse formation⁹, implying that neuexins function in the specification and initiation of synapse formation. The intracellular tails of

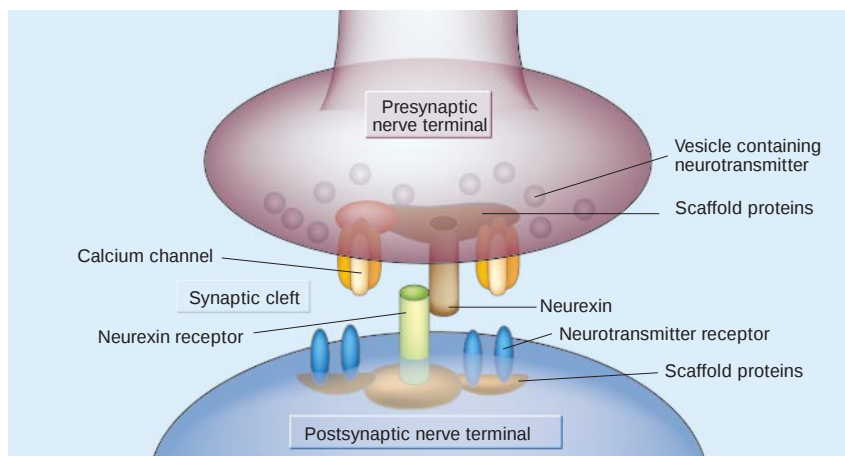


Figure 1 A neuexin-based adhesion complex in synapses. In this model, which incorporates the findings of Missler *et al.*¹, presynaptic neuexins bind across the synaptic cleft to specific receptors in the postsynaptic membrane, thereby aligning presynaptic and postsynaptic apparatuses. In the presynaptic neuron, intracellular scaffold proteins that bind to the tail of neuexin mediate the clustering or functional activation of calcium channels in close proximity to neurotransmitter-containing vesicles and the neurotransmitter-release machinery. Meanwhile, postsynaptic scaffold proteins that bind to the neuexin receptor organize postsynaptic clusters of neurotransmitter receptors.

neurexin and neuroligin bind respectively to CASK and PSD-95, two 'scaffold' proteins that are believed to organize additional proteins of the presynaptic and postsynaptic membranes and link them to the neuron's internal skeleton (the cytoskeleton)¹⁰ (see Fig. 1). This series of protein interactions could enable a neurexin–neuroligin complex to glue together presynaptic and postsynaptic elements.

These protein-interaction and *in vitro* studies are revealing — but what do neurexins do in living organisms? To find out, Missler *et al.*¹ generated 'knockout' mice in which one, two or all three α -neurexin genes were disrupted. The triple-knockout mice were alive at birth, but rapidly died of respiratory failure. Surprisingly, however, the brains of these animals did not show any obvious anatomical defects, except for a twofold reduction in the number of inhibitory synapses. Moreover, synapses appeared structurally normal by electron microscopy — implying that neurexins are not, after all, essential for synapse formation.

Electrophysiological analysis, however, revealed a profound impairment in neurotransmitter release in the triple-knockout mice. Transmitter release was readily evoked by drugs that bypass presynaptic calcium channels, implying that the release machinery itself was intact. Rather, the fault appeared to lie with the calcium channels, which showed a severe loss of activity in neurexin-deficient neurons, despite being present at normal density on the cell surface. Provocatively, this defective calcium-channel function was seen only after the developmental period encompassing synapse formation, not before, hinting that channel activity was lost during synapse formation in the absence of neurexins.

This genetic analysis has shifted our view of neurexins: it seems that they are not needed for synapses to form or to achieve specificity of neuronal connections. Instead, Missler *et al.* propose that neurexins are essential for the molecular and functional integration of calcium channels with the basic presynaptic release machinery. Moreover, by binding 'trans-synaptically' to specific postsynaptic proteins, neurexins could offer a simple means of restricting the activity (and/or localization) of presynaptic calcium channels to specific sites (Fig. 1).

As usual, several key questions remain. For instance, what are the relevant postsynaptic protein partners of α -neurexins, and do they modulate neurexin's effects on calcium channels? Given the surprising ability of a single form of neurexin to largely restore the knockout mice to normality¹, what is the significance of the prolific alternative splicing of neurexins (which regulates their interactions with both neuroligin and dystroglycan)? Are presynaptic calcium channels mislocalized, or are they only

functionally impaired, in the absence of neurexin? Do the smaller, mammalian-specific β -neurexins — which bind neuroligins — have further roles in synapse formation and organization? Finally, what are the signals that regulate the function of presynaptic calcium channels, ultimately ensuring that calcium influx only occurs through those channels that are present at sites defined by the α -neurexin trans-synaptic adhesion complex? The answer to this question could be relevant to how we learn and remember information, as such signals might be substrates for altering the strength of synaptic transmission — an essential determinant of learning and memory. ■

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Earth science

Spread thin in the Arctic

Emily M. Klein

The northernmost segment of the global mid-ocean-ridge system, the Gakkel ridge, is a bit of an oddity. Provocative data are emerging from an international geophysical and geochemical study of the region.

In 1893, Fridtjof Nansen and members of his expedition set out to conduct a remarkable scientific study in the Arctic. Their ship, the *Fram*, was designed to become encased in pack ice and to move with it, and over a three-year period the *Fram*'s stately motion demonstrated the drift of the polar ocean current. Just over a century later, a fresh chapter in the scientific study of the northern high latitudes opened with a two-ship, international expedition to study the ocean floor beneath the Arctic Ocean. Some results from this study — AMORE, for Arctic Mid-Ocean Ridge Expedition — appeared in *Nature* early this year¹, and others are described by Michael *et al.*² and Jokát *et al.*³ on pages 956 and 962 of this issue. The Arctic Ocean is covered by sea ice, so the studies were carried out on scientifically equipped icebreakers, the US Coast Guard Cutter *Healy* and the German PFS *Polarstern*.

At mid-ocean ridges, partial melting of upwelling mantle from deep within the Earth produces magmas that erupt and cool to become basalt lavas. This magmatic activity creates new ocean crust that spreads on either side of the ridge, at different rates depending on the location. Ridges may also be sites of intense hydrothermal activity, as sea water penetrates the crust, becomes heated by its proximity to hot magma at depth, and re-emerges into the water column through vents as element-rich fluids.

The ridge beneath the Arctic Ocean is the 1,800-km-long Gakkel ridge (Fig. 1), and it

has some unique characteristics. Given these characteristics, studies of other parts of the global mid-ocean-ridge system allowed certain predictions to be made about what would be found there. For instance, almost three decades ago aeromagnetic surveys had shown that the Gakkel ridge is spreading at the slowest rate of any mid-ocean ridge⁴, with a 'full rate' (that is, accounting for motion on either side) of about 0.3–1.0 cm per year in the eastern part. That compares with about 6 cm per year for 'intermediate-spreading' ridges elsewhere. Spreading rate had been shown to correlate in a general way with a host of ridge characteristics, such as the balance between magmatism and tectonic faulting, the composition of the lavas, the architecture of the ocean crust, and the nature and distribution of hydrothermal activity.

From these correlations, it was predicted that the Gakkel ridge would have generally 'anaemic' magmatism that would progressively decrease in volume as the spreading rate decreased towards the east, where there is a thicker, conductively cooled cap on mantle upwelling and melting⁵. In addition, the extrapolation from fast-spreading to slow-spreading rates suggested that there would be little or no hydrothermal activity along ultraslow-spreading sections such as the Gakkel ridge, because of decreased magmatism and heat (which together drive hydrothermal circulation).

Surprisingly, both of these predictions were found to be incorrect. Based on their spectacular bathymetric and tectonic maps

of the western 1,000 km of the ridge, as well as the amounts of lava recovered by dredging, Michael *et al.*² show that the eruption of magma is surprisingly robust in the western zone, only sparse in the central zone, and robust again in the eastern zone (Fig. 1), where the spreading rate is slowest. Furthermore, hydrothermal plume activity turns out to be among the most vigorous of any ocean ridge yet studied. The authors speculate that this is a consequence of the highly focused nature of the magmatism there, which leads to long-lived, discrete volcanic structures, combined with episodes of crustal extension and faulting. These are the two factors required for hydrothermal activity: faulting provides the pathway for sea water to penetrate into the sea floor, and magmatism provides the heat to drive fluid circulation.

The Gakkel ridge lies about 5 km beneath the ocean surface, which is unusually deep — about twice the average — for ocean ridges worldwide. The implication is that the crust here is unusually thin⁶, possibly as a result of a combination of cool mantle temperatures and ultraslow spreading. Using the methods of seismic refraction, Jokat *et al.*³ show decisively that the crust here is indeed exceedingly thin. In fact, dredging in the deepest parts of the central zone of the ridge recovered an abundance of rocks that are typical of the mantle, but few or no basalts — that is, basaltic crust that usually overlies mantle seems to be completely absent in some places.

Finally, the new results^{2,3} provide essential data for examining the relationship between the chemical composition of basalt lavas and the crustal thickness of the ridge from which they were collected. Figure 2 shows the inverse correlation between

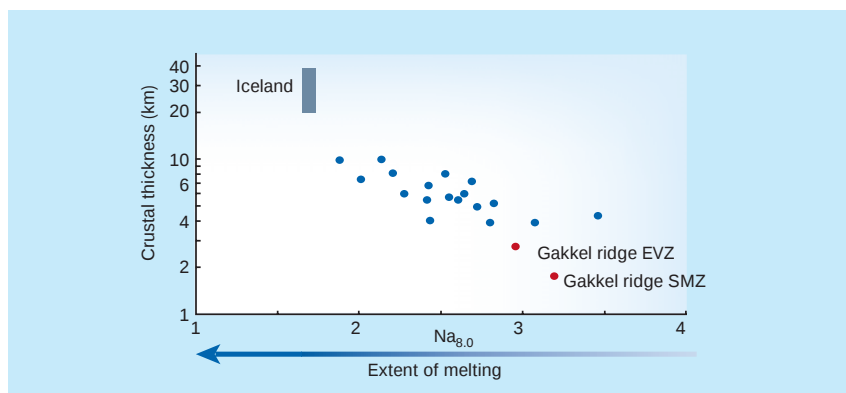


Figure 2 Correlation of the chemical composition of ocean-ridge basalt lavas with the crustal thickness of the ridge. Shown here are regional averages of the crustal thicknesses associated with various locations on mid-ocean ridges around the world, as inferred from seismic data, plotted against a measure of the sodium content of the lavas ($\text{Na}_{8.0}$, which is Na_2O normalized to 8% MgO)⁷. The new measurements^{2,3} from the Gakkel ridge — from the eastern volcanic zone (EVZ) and the sparsely magmatic zone (SMZ) — extend this correlation to the very thinnest crust yet identified. Determinations of crustal thickness are from ref. 7, augmented or superseded by data from many sources; chemical analyses are primarily from ref. 9.

regional averages of crustal thickness and the 'normalized' sodium contents of lavas recovered from ridges worldwide^{7,8}. This correlation may be explained by assuming that regional differences in the extent of mantle melting produce corresponding variations in basalt composition. Sodium is most abundant in magmas produced by small amounts of melting. And if less melt is produced, the crust created by that melt will be thinner.

The extreme position of the Gakkel ridge on Fig. 2 suggests that it does indeed represent a global 'end-member' in terms of its small extent of melting. Although most researchers agree that variations in mantle temperature lead to differences in the extent of melting from region to region, some have

argued that spreading rate may play a significant or even dominant role at ridges that spread at ultraslow rates⁵. Michael *et al.* argue that if spreading rate were the primary factor governing the extent of melting, then the normalized sodium in the lavas ($\text{Na}_{8.0}$)⁷ should increase, and magmatic output should decrease, on moving from the western volcanic zone, through the central zone, into the eastern zone as spreading rate decreases. In fact, although the western zone is lowest in $\text{Na}_{8.0}$, consistent with greatest extents of melting, the high $\text{Na}_{8.0}$ content of the central zone is equivalent to that of the more eastern zone. Here, then, is evidence against a simple relationship between spreading rate and magmatism.

These are just some of the wealth of results to emerge from studies of the Gakkel ridge. Perhaps the most important finding is that scientific preconceptions continue to be challenged with each new project in Earth science, underscoring the necessity of continued exploration of the structure, chemistry and behaviour of our planet.

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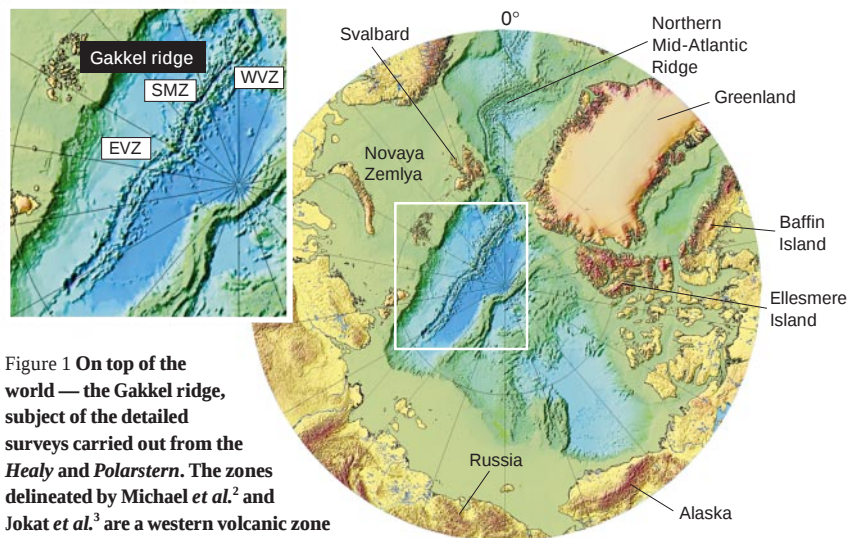


Figure 1 On top of the world — the Gakkel ridge, subject of the detailed surveys carried out from the *Healy* and *Polarstern*. The zones delineated by Michael *et al.*² and Jokat *et al.*³ are a western volcanic zone (WVZ); a central, sparsely magmatic zone (SMZ); and an eastern volcanic zone (EVZ). Only the western 1,000 km of the 1,800-km ridge were accessible for study. Brown is land; light green, continental shelf; blue, deep ocean.

Obituary

Jeff Schell (1935–2003)

What can we learn from a soil bacterium that induces tumours in plants? This was the question that Jeff Schell set out to tackle when, in 1967, he became an associate professor of genetics at what was then called the Rijksuniversiteit Gent. Schell died on 17 April 2003. In the intervening quarter-century, the lessons that he and others extracted from work with the bacterium concerned, *Agrobacterium tumefaciens*, were to provide the basis for the plant biotechnology industry.

In the mid-1960s, it had just become generally accepted that some viruses cause tumours, as was acknowledged with the award of a 1966 Nobel prize to Peyton Rous. But bacteria capable of inducing tumours in animals were unheard of. And although there had long been an awareness of plant tumours called 'crown galls', they generated only minor research interest. Schell, however, wanted to explore at the molecular level what, 20 years previously, Armin Braun had dubbed "tumour-inducing property": the ability of *Agrobacterium* to induce crown galls.

Schell had been trained in Gent as a bacterial taxonomist, and had then had the good fortune to do postdoctoral work with Bill Hayes, the father of bacterial genetics, at Hammersmith Hospital in London. Later, with Lou Siminovitch in Toronto, he discovered the fascinating world of bacteriophage — viruses that infect bacteria. Along with this grounding in bacterial and phage genetics, he had an especial talent for formulating hypotheses and designing experiments to test them. All of this was the perfect background for solving the crown gall puzzle.

Jeff and I had both been active in the same student movements. When he became a professor, his lab was next to mine, and we decided to join forces. A good collection of oncogenic and non-oncogenic strains of *Agrobacterium* was available to us at the taxonomy laboratory where he had done his PhD, and we set to work comparing the strains. We found that tumour-inducing strains of *Agrobacterium* harboured a large element, which we called the Ti-plasmid. And we proposed that some DNA of this Ti-plasmid might become integrated into the genome of its host and induce the crown gall. This hypothesis met with scepticism from most plant physiologists as a seemingly wild and untestable idea — of course, molecular biology was then only just coming into existence.



Pioneer of plant genetic engineering

It was not long, however, before the appropriate techniques were developed. With the advent of gene cloning and DNA hybridization by Southern blots, proof came fast that a copy of a segment on the Ti-plasmid was preferentially inserted in expressed DNA sequences of the plant. But why were genes in a bacterium active only in a plant cell?

The answer stemmed from work by Georges Morel and Jacques Tempé, who had shown that the Ti-plasmid causes a plant to produce certain unique amino-acid-derived molecules. From this, Schell developed the concept of 'genetic colonization', according to which the whole *raison d'être* of the Ti-plasmid is making plant cells secrete these molecules, which constitute a source of carbon and nitrogen that only *Agrobacterium* can use. Crown galls, then, constitute a unique ecological niche for the microbe.

The implication of these findings was clear: if DNA was being transferred by *Agrobacterium*, it should be possible to replace the tumour-inducing genes by others that conveyed new traits to plants. The race to turn *Agrobacterium* into a reliable gene vector was on, with fierce competition from Gene Nester at the University of Washington in Seattle, and Mary-Dell Chilton at Washington University and Monsanto in St Louis. Schell was a keen sportsman, with a particular interest in sailing, and he relished competition (especially when he felt he could win). Nonetheless, this was a scientific race conducted on amicable

terms, with information being exchanged and synchronized publication of many of the notable papers.

In 1978, Schell had become director of the Max Planck Institute for Plant Breeding Research in Cologne, Germany. This he turned into an international leader in plant research, nurturing generations of students and young scientists from around the world. He continued co-directing the team in Gent, however, and our work culminated in 1983 with the announcement, simultaneously with Chilton and Monsanto, of the *Agrobacterium*-mediated creation of the first genetically modified plants that expressed a new trait.

With this development, plant molecular biology had a key tool with which to flourish: from then on, transgenes and gene regulatory sequences could be routinely transferred into plants. Financiers and agrochemical companies became keen to invest in the new discipline of plant biotechnology through start-up companies focusing on crop improvement. Schell was instrumental in helping many of these industrial initiatives to integrate the findings of fundamental research into their activities. Throughout his career he was concerned about injustice and suffering; he was particularly alarmed by environmental problems, and was convinced that this new technology would be instrumental in achieving more sustainable agricultural practices. He believed that, for all their good intentions, those who wish to ban genetic modification in crops, or who argue that developing countries should reject food-aid with a genetically modified component, are profoundly misguided.

Even though Schell was suffering from a neurodegenerative disease, right up to his death he continued to put the case for the need to apply plant biotechnology to help farmers in developing countries. He was also particularly active in promoting German–Israeli cooperation in the plant sciences, through the Otto Warburg Minerva Center in Jerusalem, which runs joint programmes with the Weizmann Institute and the Hebrew University.

Jeff Schell was not only a thoughtful and innovative scientist, but also an outstanding speaker and a charismatic mentor. And in one sense, the best may be yet to come, for the enduring influence of his work is likely to be seen in many of the advances in agriculture in the decades ahead.

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Materials science

Nanoparticles target tumours

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Photodynamic therapy is an increasingly common treatment for tumours.

Light-sensitive drugs are delivered to a tumour, which is then irradiated to excite the drug molecules. Molecular oxygen inside the tumour picks up the excess energy, forming reactive species such as singlet oxygen that cause irreversible damage to the tumour cells.

Indrajit Roy *et al.* have tested ceramic-based nanoparticles as drug carriers. Unlike the molecular aggregates currently in use, they do not release the drugs, but have a porous matrix that is permeable to both molecular and singlet oxygen. The nanoparticle carriers are highly stable, and are therefore more likely to reach the tumour without release or denaturation of the drug en route — a major problem with aggregate particles.

The authors found that, *in vitro*, the ceramic particles were actively taken up by tumour cells, and the survival rate of these cells was much lower than that of similar cells that were irradiated but untreated. So more efficient photodynamic treatment, using ceramic nanoparticles as drug carriers, could be in sight: research is now under way *in vivo*.

Jane Morris

Neurobiology

Wiring the brain

Neuron **38**, 887–898 (2003)

Mutations in the fragile X mental retardation protein (FMRP) or in proteins similar to Rac1 both cause mental retardation in humans. Now Annette Schenck and colleagues have found that another protein implicated in the condition, called CYFIP, may represent a hitherto missing link between FMRP and Rac1, and so affect brain wiring.

Schenck *et al.* found that when fruitfly embryos are genetically engineered to lack a working copy of CYFIP, their neurons are shortened and grow in the wrong direction. Using genetic and biochemical techniques, the authors discovered that CYFIP usually interacts with both FMRP and Rac1. They suggest that, under normal circumstances, the three proteins form a pathway that controls the growth of the cytoskeleton inside nerve axons. This fits with the finding that in people with fragile X syndrome — one of the commonest forms of mental retardation — the number and shape of the delicate fingers linking nerve cells appear abnormal.

One possibility is that Rac1, when

activated by unknown signals outside the neuron, links up with CYFIP and that this releases active FMRP. Both are then thought to influence neuronal structure: Rac1 affects the assembly of a cytoskeletal building-block called actin, and FMRP controls the production of other structural proteins.

Helen Pearson

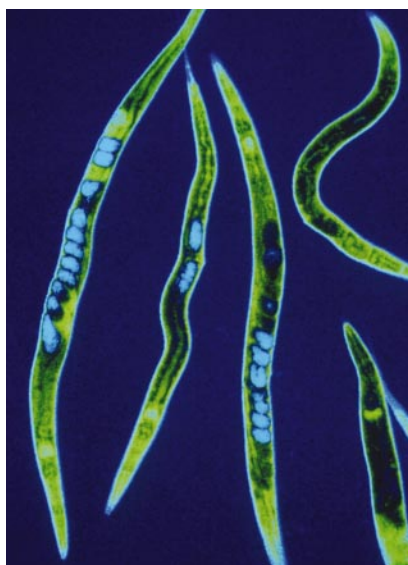
Evolutionary biology

Compensation claim

Evolution **57**, 1022–1030 (2003)

Small populations that are not subject to natural selection will accumulate deleterious mutations. But put them back in a competitive environment and they may regain fitness surprisingly rapidly, find Suzanne Estes and Michael Lynch.

The authors studied laboratory lines of the nematode *Caenorhabditis elegans* (pictured). These lines had been maintained



by allowing only one individual from each generation to reproduce, and in the absence of competition they became less fit than their counterparts in the wild. Competition was reinstated by allowing groups of 10,000 (or more) worms from each line to breed in each generation. After just a few dozen generations, their rates of survival and progeny production — both measures of fitness — had increased.

Estes and Lynch attribute this recovery to compensatory mutations that ameliorate the influence of the deleterious mutations. Control populations did not increase in fitness under the same conditions, showing that the effect is not due to generally beneficial mutations.

There could be a happy message here for captive breeding of threatened species, given the worries about loss of genetic vigour in such programmes — although, of course, most endangered species breed much more slowly than nematodes.

Michael Hopkin

Astrophysics

Star spins flat out

Astron. Astrophys. (in the press); preprint astro-ph/0306277 at <http://arXiv.org> (2003)

A team of astronomers has found a flattened star. Achernar (or α Eridani), in the constellation of Eridanus, is rotating so rapidly that it appears squashed, resembling a child's spinning-top. The diameter at its equator is 8.4 million kilometres (12 times the width of the Sun), but the pole-to-pole distance is, at most, 5.4 million kilometres. If we are not seeing Achernar perfectly 'edge-on', its flatness is even more pronounced.

The squished star was spotted by A. Domiciano de Souza and co-workers, using the Very Large Telescope Interferometer in northern Chile. Six times as massive as the Sun, Achernar is 145 light years away. Measuring its shape from Earth is equivalent to measuring the dimensions of a motor car on the surface of the Moon.

As they are essentially balls of hot gas, stars are relatively easily deformed by rotation. But seeing a star this 'flat' is quite a surprise. Either it is rotating close to the break-up speed of 300 km s⁻¹, or it is not rotating as a 'rigid' body.

Philip Ball

Cell biology

Flies get rhythm

Cell **113**, 755–766 (2003)

Whole organisms, tissues and cells can all have rhythm — patterns of behaviour, physiology and gene expression that occur in a roughly 24-hour cycle. These patterns are generated by intracellular clocks, which are regulated by key genes. In many fruitfly tissues, for instance, the *Clock* gene triggers the expression of so-called circadian genes, which in turn regulate their own production in a negative-feedback loop. This generates a clock-like cycle of gene activity and inactivity.

In the adult fly brain, neurons that express *Clock* are the exception rather than the rule. But Jie Zhao *et al.* now find that forcing the expression of just this gene can bring rhythm to neurons that have none. Misexpressing *Clock* in brain regions where it is not normally active prompted the expression of circadian genes such as *timeless*, as well as *cryptochrome*, which encodes a photoreceptor for blue light.

The flies also behaved differently. Normally, they show two bursts of activity as lights go on and off. Some of the mutant female flies, however, were active all 'day' and moved little at 'night'. This suggests that the new clocks make functional connections with behavioural pathways, lending weight to the idea that *Clock* is a master regulator of circadian rhythms.

Helen R. Pilcher

Facial expressions linked to monkey calls

Pulling a face to emphasize a spoken point is not seen as just a human prerogative.

The perception of human speech can be enhanced by a combination of auditory and visual signals^{1,2}. Animals sometimes accompany their vocalizations with distinctive body postures and facial expressions³, although it is not known whether their interpretation of these signals is unified. Here we use a paradigm in which 'preferential looking' is monitored to show that rhesus monkeys (*Macaca mulatta*), a species that communicates by means of elaborate facial and vocal expression⁴⁻⁷, are able to recognize the correspondence between the auditory and visual components of their calls. This crossmodal identification of vocal signals by a primate might represent an evolutionary precursor to humans' ability to match spoken words with facial articulation.

We tested whether rhesus monkeys could recognize auditory–visual correspondence between their 'coo' and 'threat' calls (Fig. 1a). These are the most frequently produced calls in the rhesus monkey repertoire, both in the wild and in captivity. Coo calls are long, tonal signals (Fig. 1b) and are produced in an affiliative context⁵; threat calls, by contrast, are noisy, pulsatile calls of short duration (Fig. 1b) and are produced during agonistic encounters⁵. Each of these calls is accompanied by a unique facial gesture: coo calls are associated with a small mouth opening and protruding lips, whereas threat calls are conveyed with a larger mouth opening and no lip protrusion^{6,7} (Fig. 1a).

To test the monkeys, we adopted a preferential-looking technique that has been used to test crossmodal speech perception in prelinguistic human infants^{8,9}. Subjects were seated in front of two liquid-crystal-display monitors and shown two side-by-side digital 2-s videos (synchronized to the onset of the audio track), played in a continuous loop for 1 min, of the same conspecific individual ('stimulus animal') articulating the two different calls. A sound track that corresponded to one of the two facial postures was played through a speaker located behind and between the two monitors; a curtain concealed all equipment apart from the screens and the camera lens. Each trial began when the subject fixated his gaze towards the camera, and was scored by two observers who were blind to the conditions and who judged, frame by frame, whether the subject was looking towards the right screen, the left screen or away from both (inter-observer agreement was 0.98, as measured by Pearson's correlation).

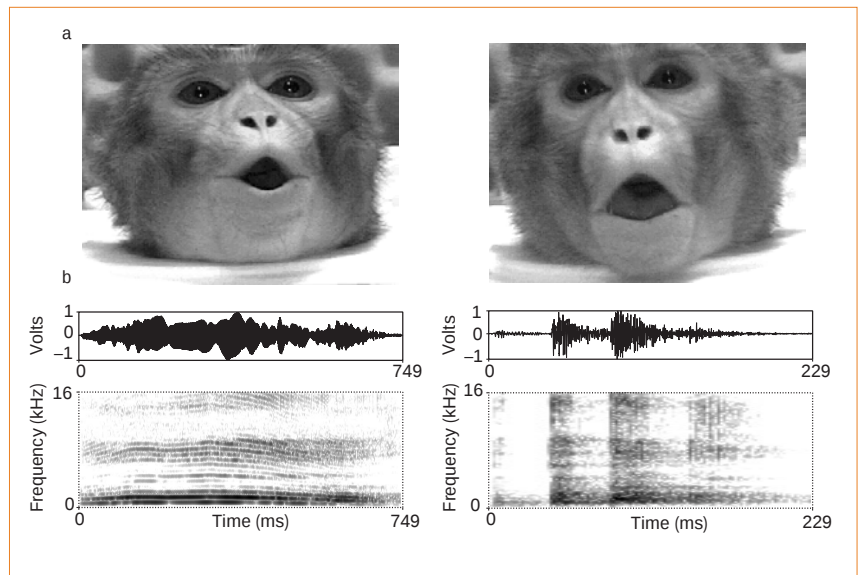


Figure 1 Multisensory integration by rhesus monkeys of their 'coo' and 'threat' calls. **a**, Single video frames of facial gestures made during coo (left) and threat (right) calls, shown at the point of maximal mouth opening. **b**, Oscillograms (top) and spectrograms (bottom) of the coo and threat vocalizations: coo calls are long and tonal; threat calls are short, pulsatile and noisy.

We used two separate stimulus sets with two different stimulus animals to ensure that recognition was not based on an idiosyncratic property of the vocalizer. The call played through the speaker, the left–right position of the two facial gestures, and the stimulus sets were counterbalanced. The dependent measures used were the percentage of total looking time to the match video, and the duration of the longest single look at each of the two screens.

We predicted that, if rhesus monkeys recognized the correspondence between the heard sound and the appropriate facial posture, then they would spend more time overall looking at the match video. We found that the mean percentage of looking time devoted to the match was 67.3%, which was significantly greater than the 50% chance value ($t = 4.19$, d.f. = 10, $P = 0.002$). Furthermore, all 11 subjects looked for longer at the match face (binomial test, $P < 0.001$). There were no significant interactions between the time spent looking at the match face and the stimulus set or the position (left versus right) of the monitor. Subjects did, however, look for longer when the match face was articulating the coo call than when the match face was making the threat call ($F = 18.75$, d.f. = 10, $P = 0.002$). A separate analysis revealed that preferences for looking to the match screen were still significant for coo calls alone ($t = 6.20$, d.f. = 4, $P = 0.003$) and threat calls alone ($t = 2.98$, d.f. = 5, $P = 0.03$). Rhesus monkeys' longest

single fixations were, on average, longer for the match (mean $\pm$ s.e.m., 7.97 ± 1.16 s) than for the non-match face (4.26 ± 0.98 s). This difference was significant ($t = 3.94$, d.f. = 10, $P = 0.003$). Furthermore, 10 of the 11 subjects had longer single fixations to the match face (binomial test, $P = 0.006$).

Our results indicate that rhesus monkeys have an inherent ability to match acoustically presented conspecific vocalizations with the appropriate facial posture. The pattern of our results closely follows those for crossmodal speech recognition by prelinguistic human infants using the same preferential-looking paradigm^{8,9}. However, to our knowledge, this is the first demonstration of auditory–visual integration in an animal vocal communication system. We interpret the preferential fixations on the sound-specified facial expression as evidence that rhesus monkeys detect information that is invariant across visual and acoustic displays of vocal expressions. The presence of multimodal perception in an animal's communication signals may represent an evolutionary precursor of humans' ability to make the multimodal associations necessary for speech perception^{10,11}.

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Biomechanics

Bacterial flagellar switching under load

Flagellated bacteria swim up gradients of chemical attractants by modulating the direction of rotation of their flagellar motors, but how this switching mechanism works is not understood. Here we show that the probability of the motor rotating in the clockwise direction increases under high load, when the motor spins slowly (at less than 50 Hz). We suggest that either the switch is responding to small changes in torque — the torque increases only fractionally between 50 Hz and stall — or it senses motor speed, perhaps by means of proton flux.

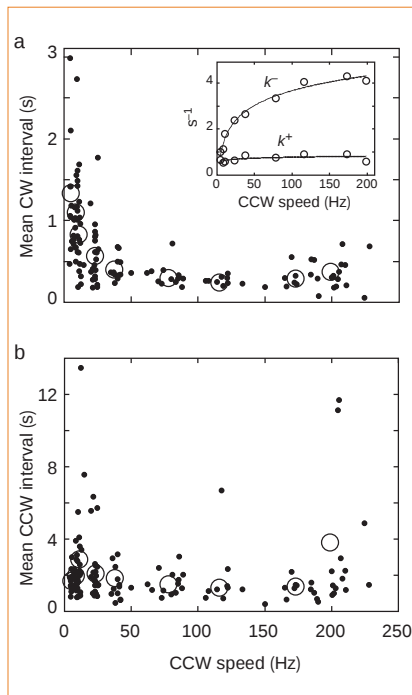


Figure 1 Behaviour of the flagellar switch of *Escherichia coli* under different loadings. **a**, **b**, Mean intervals for clockwise (CW; **a**) and counter-clockwise (CCW; **b**) rotation of the flagellar motor, plotted as a function of mean CCW speed. Filled symbols, values measured for each cell; hollow symbols, averages over the ensemble of cells for beads of a given size, with the values for each cell being weighted equally. Bead diameters were 0.36, 0.54, 0.74, 1.03, 1.20, 1.44, 1.79, 2.13 and 2.60 μm (from right to left); the number of cells studied for each of these bead sizes was 12, 12, 7, 12, 12, 18, 18, 16 and 10, respectively. Each bead was monitored for 5 min at 20 $^{\circ}\text{C}$. Inset, rate constants $k^- = 1/(\text{mean CW interval})$ and $k^+ = 1/(\text{mean CCW interval})$ were computed for each cell and then averaged over the ensemble of cells for beads of a given size, with the values for each cell being weighted equally.

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Competing financial interests: declared none.

Flagellar filaments of an *Escherichia coli* sticky-filament mutant¹, which is otherwise wild-type for chemotaxis, were shortened by viscous shear, and cell bodies were fixed to a polylysine-coated glass coverslip. Latex beads of various sizes were adsorbed to filament stubs and their rotation was followed in a weak optical trap with a quadrant detector².

Figure 1 shows clockwise and counter-clockwise intervals for rotation plotted as a function of counter-clockwise speed, together with rate constants for transitions between the clockwise and counter-clockwise states. Clockwise intervals lengthened appreciably at high loading (k^- decreased), whereas counter-clockwise intervals remained about the same for all loads (k^+ remained roughly constant).

A similar asymmetrical performance is seen under the action of fumarate³. However, when an attractant is added, the cells respond by shortening their clockwise intervals and lengthening their counter-clockwise intervals⁴. From the results shown in Fig. 1, it follows that a motor that is spinning rapidly, as motors do in swimming cells, will spend a larger fraction of its time turning counter-clockwise and will change direction more frequently than a motor that is spinning slowly, as motors do in tethered cells.

In a wild-type cell, the direction in which the motor spins depends on the degree of phosphorylation of a small signalling protein, CheY, which is activated by a kinase that is coupled to the chemoreceptors⁵. To determine whether this signalling pathway is involved in the load response, we repeated a number of measurements using cells in which this pathway had been disrupted. We used a strain in which *cheY*, *cheA* (which encodes the kinase) and *cheZ* (which encodes a phosphatase) were all deleted and which expressed CheY^{13DK106Y} (CheY**), a variant with fixed activity¹. The response to different loadings was the same as that of wild-type cells, indicating that the motor's sensitivity to load is probably not due to feedback from the chemotaxis signalling network, but is instead an inherent property of the motor.

The bacterial flagellar motor is powered by protons moving down an electrochemical gradient and has a distinctive torque–speed relationship. At 20 $^{\circ}\text{C}$, the relative torque falls gradually from unity at stall to about 0.9 at 120 Hz, and drops rapidly to zero at about 260 Hz (ref. 6); in the latter regime, the torque decreases

markedly at lower temperatures or when H_2O is replaced by D_2O (ref. 7), indicating that the movement of mechanical parts and/or of protons becomes rate limiting.

We found switching to be particularly sensitive to load only during high-torque, low-speed operation, and mainly at speeds of less than about 50 Hz (Fig. 1). Over this speed range, the torque changes by about 5%. There is evidence that each revolution of the motor requires the passage of a fixed number of protons⁸. So, rather than sensing changes in torque, might the switch be monitoring proton flux?

Flagellated bacteria are sensitive to a variety of environmental factors, including mechanical stimuli. Swimming in viscous solutions or near surfaces can trigger developmental changes — for example, swarmer-cell differentiation in *E. coli*⁹ or lateral flagellar synthesis in the marine organism *Vibrio*¹⁰. In the latter case, changes in gene expression occur in response to exposure to sodium-channel blockers, indicating that they might be caused by a reduction in motor ion flux¹¹. It is possible that these different responses rely on a common mechanosensory mechanism.

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Competing financial interests: declared none.

erratum

Rapid change in mouse mitochondrial DNA

Oliver R. W. Pergams, Wayne M. Barnes, Dennis Nyberg
Nature **423**, 397 (2003)

There is an error in the Volo Bog and Glenview components of Fig. 1 of this communication: two *Mw* haplotype mice were shown in the 1950–99 column of the former that should have been in the 1950–99 column of the latter. This mistake does not affect the conclusions, as the analysis was done with mice from the correct category. Sample sizes (left and right columns, respectively) for the different locations were: Volo Bog, 19 and 41; Glenview, 5 and 6; Illinois Beach, 8 and 5; Highland Park, 2 and 5; Palos, 1 and 8 (note that sample numbers were inevitably low for museum specimens).

α -Neurexins couple Ca^{2+} channels to synaptic vesicle exocytosis

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Synapses are specialized intercellular junctions in which cell adhesion molecules connect the presynaptic machinery for neurotransmitter release to the postsynaptic machinery for receptor signalling. Neurotransmitter release requires the presynaptic co-assembly of Ca^{2+} channels with the secretory apparatus, but little is known about how synaptic components are organized. α -Neurexins, a family of >1,000 presynaptic cell-surface proteins encoded by three genes, link the pre- and postsynaptic compartments of synapses by binding extracellularly to postsynaptic cell adhesion molecules and intracellularly to presynaptic PDZ domain proteins. Using triple-knockout mice, we show that α -neurexins are not required for synapse formation, but are essential for Ca^{2+} -triggered neurotransmitter release. Neurotransmitter release is impaired because synaptic Ca^{2+} channel function is markedly reduced, although the number of cell-surface Ca^{2+} channels appears normal. These data suggest that α -neurexins organize presynaptic terminals by functionally coupling Ca^{2+} channels to the presynaptic machinery.

Synapses are composed of precisely aligned pre- and postsynaptic specializations that are thought to be connected by synaptic cell-adhesion molecules. Synaptic transmission varies between synapses, and changes as a function of use. For example, the probability of neurotransmitter release and the magnitude of short-term plasticity differ among synapses^{1–3}. Even presynaptic terminals formed by a single neuron exhibit distinct release probabilities that are instructed by the postsynaptic neuron⁴. Key determinants of presynaptic function are voltage-gated Ca^{2+} channels that mediate the influx of Ca^{2+} , which then triggers release. These channels may be physically connected to the secretory machinery^{5–7}. Presynaptic Ca^{2+} channel activity varies among synapses, and can differ even between synapses formed by the same neuron, implying that postsynaptic signals modulate presynaptic Ca^{2+} channels⁸. Although Ca^{2+} channels are well characterized⁹, we know little about the machinery that controls the function of Ca^{2+} channels at a synapse. Synaptic cell-adhesion molecules probably nucleate assembly of pre- and postsynaptic specializations, and may link Ca^{2+} channels to secretory components by interacting with synaptic PDZ domain proteins, SNAREs (soluble NSF attachment protein receptors), and synaptotagmin^{7,10,11}. Candidates for this role as synaptic cell-adhesion molecules are neurexins^{12–15}. Neurexins are highly polymorphic cell-surface proteins that are localized on presynaptic terminals, where they act as receptors for α -latrotoxin, a neurotoxin that triggers neurotransmitter release^{12,13}. As presynaptic cell-adhesion molecules, neurexins bind to the postsynaptic cell-adhesion molecules dystroglycan¹⁴ and neuroligins¹⁵, which are thought to perform important functions in shaping synapses^{16–18}. Intracellularly, neurexins interact with the PDZ domain proteins CASK¹⁹ and Mints²⁰, and the synaptic vesicle protein synaptotagmin²¹, all of which may also modulate Ca^{2+} channels^{7,11}. The polymorphic nature and binding activities of neurexins indicated that they act as cell-recognition and cell-adhesion molecules at the synapse²². However, the evidence for a synaptic function of neurexins is indirect. Using knockout (KO) mice, we show that α -neurexins are essential for the functional organization of synapses where they are required for the activity of presynaptic Ca^{2+} channels, suggesting a new molecular pathway that links synaptic cell adhesion to the machinery for synaptic transmission.

α -Neurexins are essential for survival

In vertebrates, neurexins are encoded by three large genes (*Nrxn1*, *Nrxn2*, *Nrxn3*) of up to 1.6 megabases (Mb)²³. Each gene contains separate promoters for α - and β -neurexins. As only α - but not β -neurexins are evolutionarily conserved²³, we concentrated on α -neurexins, and targeted all three isoforms because of the potential for redundancy. Using homologous recombination, we generated mice in which the large first coding exons of all three α -neurexin genes were removed (see Supplementary Fig. S1). Immunoblotting of brain proteins with an antibody that recognizes all α - and β -neurexins confirmed that the KOs deleted expression of α -neurexins without significant effects on β -neurexins (Fig. 1a).

Deletion of α -neurexins did not impair prenatal viability, but markedly compromised postnatal survival (Fig. 1b). We detected all mutant genotypes in the expected mendelian ratios when we analysed mice immediately before birth by Caesarean section (data not shown). All double and triple KO mice were alive at birth and breathed, moved and reacted to tactile stimuli. However, all triple KO mice died on the first day, most double KO mice perished within the first week, and even single neurexin 3 α KO mice exhibited impaired survival (Fig. 1b). This suggests that neurexins are essential but redundant, consistent with the co-expression of multiple neurexins in neurons²⁴. However, the functions of α -neurexins only partly overlap because different combinations of α -neurexin KOs induced distinct mortality rates (Fig. 1b). The essential nature of α -neurexins made breeding of α -neurexin KO mice difficult. Thus, to study mice that lack two or three α -neurexins but have the same genetic backgrounds, we often used heterozygous or single KO mice as littermate controls for double and triple KO mice (see Methods).

α -Neurexin-deficient mice, especially double and triple KO mice, breathed with difficulty. Using whole-body plethysmography, we found that respiration was abnormal in single and double KO mice, and severely impaired in triple KO mice (Fig. 1c; see also Supplementary Fig. S2). The respiratory rhythm, measured as the coefficient of variation of respiration, was highly irregular in the KO mice (wild type, 0.09; neurexin 2 α KO, 0.15; neurexin 2 α /3 α double KO, 0.26; neurexin 1 α /2 α double KO, 0.43; triple KO, 0.59; normal range in newborn mice, 0.1–0.2). Recordings from

rhythmically active brainstem slices revealed that the respiratory impairment in the mutant mice was an intrinsic property of the brainstem (Supplementary Fig. S2). Thus, although α -neurexin KO mice were still capable of directed movements, the integrative brain functions required for breathing were impaired with a severity that increased with the number of mutant alleles.

Brain structure in α -neurexin KO mice

α -Neurexin KO mice presumably die because essential neuronal networks involved in breathing are dysfunctional. This phenotype could in principle be caused by abnormalities in brain development,

for example, defects in neuronal migration or axonal pathfinding, or by neuronal degeneration. The following evidence, however, suggests that such changes do not explain the α -neurexin KO phenotype.

At birth, the body and brain weights of triple KO and control mice were indistinguishable (Supplementary Table S1). Mice lacking all α -neurexins exhibited no anatomical alterations in Nissl-stained serial sections, no evidence of axonal path-finding defects or increased apoptosis, and no changes in the distribution of synaptic proteins (Supplementary Figs S3 and S4). Quantifications of the levels of 22 neuronal proteins in mice lacking all α -neurexins also detected no major changes (Supplementary Table S2).

As α -neurexins are concentrated in presynaptic nerve terminals, we analysed the ultrastructure of synapses in the KO mice. We studied brainstem synapses in triple KO mice because brainstem synapses mature early, and because the breathing abnormalities in the KO mice suggested a dysfunction of the brainstem. In addition, we examined neocortical synapses in adult α -neurexin 1 α /2 α double KO mice to test whether the observations in the neonatal brainstem could be extended to the mature neocortex.

In the brainstem of triple KO mice, asymmetric type I and symmetric type II synapses were ultrastructurally normal. We found no obvious differences between wild-type and KO mice in active zone length, vesicle numbers, or vesicle size, and the width of the synaptic cleft was unchanged (wild type, 14.05 ± 1.54 nm; triple KO, 14.29 ± 1.87 nm). However, the density of symmetric (presumptive inhibitory) synapses was reduced in triple KO mice, whereas the density of asymmetric (presumptive excitatory) synapses was not (Fig. 1d). Similar observations were made in the neocortex from adult double KO mice (Fig. 1e). To validate these findings with an independent method, we labelled brainstem sections with antibodies to vesicular glutamate and GABA (γ -aminobutyric acid) transporters^{25–27}. Quantitative analysis confirmed that the relative density of GABA-releasing (GABAergic) terminals was reduced by about twofold in triple KO mice, whereas the density of glutamatergic terminals was unchanged (Fig. 1f; see also Supplementary Fig. S5). As neurexin messenger RNAs are expressed in glutamatergic and GABAergic neurons²⁴, the selective change in GABAergic synapses cannot be explained by a restricted distribution of α -neurexins. Thus the decrease in GABAergic synapses could either be due to enhanced vulnerability of GABAergic synapses in early development^{28,29}, or α -neurexins may have a general function in synapse formation that is selectively redundant in glutamatergic but not GABAergic synapses.

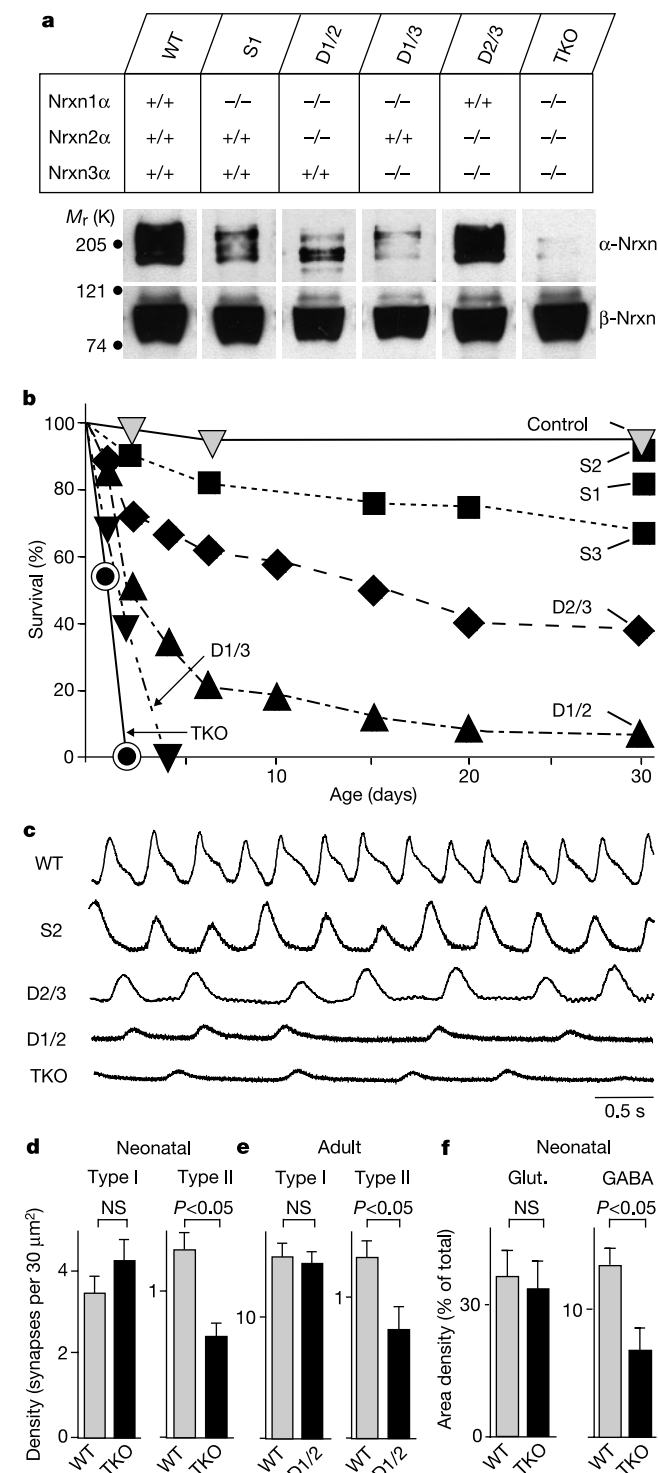


Figure 1 Essential role of α -neurexins. **a**, Immunoblot analysis of α - and β -neurexins in wild-type and α -neurexin KO mice (in this and subsequent figures, genotypes are identified as follows: WT, wild type; S1, S2 and S3, single α -neurexin 1 α , 2 α and 3 α KO mice, respectively; D1/2, D1/3 and D2/3, double KO mice of α -neurexin 1 α /2 α , 1 α /3 α and 2 α /3 α , respectively; TKO, triple KO mice). Neurexins were enriched from brain homogenates of newborn mice by glutathione S-transferase (GST)-CASK pull-downs²³, and equivalent amounts (15 μg protein per lane) were analysed by SDS-PAGE and immunoblotting with a pan-neurexin antibody. Numbers on the left indicate positions of relative molecular mass (M_r) markers. **b**, Survival of α -neurexin KO mice expressed as per cent of the expected survival based on mendelian inheritance ($n = 128$ –998 mice per time point, first time point is 8–12 h after birth). **c**, Representative respiratory traces of newborn wild-type and α -neurexin mutant mice measured by plethysmography. **d**, **e**, Density of type I (glutamatergic) and type II (GABAergic/glycinergic) synapses in the brainstem from newborn wild-type and triple KO mice (**d**), and in the neocortex from adult wild-type and α -neurexin 1 α /2 α double KO mice (**e**). Synaptic densities were measured by electron microscopy (see Supplementary Figs S3 and S4). **f**, Density of glutamatergic and GABAergic brainstem synapses in wild-type and triple KO mice measured by immunocytochemistry with antibodies to the vesicular GABA and glutamate transporters (Supplementary Fig. S5). Error bars indicate s.e.m. ($n = 3$ mice per genotype). NS, nonsignificant.

Decreased spontaneous neurotransmitter release

To test whether synapse function is impaired in α -neurexin KO mice, we analysed synaptic transmission in newborn mice using two different systems: cultured slices from neocortex and acute slices from brainstem. This double approach ensures that the phenotypes observed are independent of a particular brain region or type of synapse. We examined cultured neocortical slices because after 4–6 weeks in culture, these slices contain mature synapses that can be analysed at high resolution (Supplementary Fig. S6)³⁰. We analysed acute brainstem slices containing the pre-Bötzing complex³¹ because the brainstem provides an accessible neuronal network that is essential for normal breathing (which is disrupted in α -neurexin KO mice) and can be analysed for many different conditions (Supplementary Fig. S7).

In both preparations, we first monitored spontaneous miniature postsynaptic currents (minis) using whole-cell recordings in the presence of tetrodotoxin (a Na^+ channel inhibitor) to block action-potential-dependent network activity. In cultured slices from neocortex, GABA_A- and AMPA (α -amino-3-hydroxy-5-methyl-4-

isoxazole propionic acid)-receptor-mediated minis were separately recorded after pharmacological isolation (Fig. 2a, b). We compared triple KO mice lacking all α -neurexins with control littermate mice lacking only α -neurexin 2 α because these mice exhibited the least mortality among α -neurexin KO mice (Fig. 1b). Deletion of all α -neurexins caused a large decrease in the frequency of both GABA_A- and AMPA-receptor-mediated minis (Fig. 2c), suggesting that α -neurexins are essential for normal neurotransmitter release. We detected no change in mini amplitude, indicating that the amount of transmitter released per vesicle and the properties of postsynaptic receptors were not grossly altered. The decline in the density of neocortical inhibitory synapses (Fig. 1e) may have contributed to the decrease in the frequency of GABA_A-receptor-dependent minis. However, the equally large decrease in AMPA mini frequency (Fig. 2c) without a change in the density of excitatory, glutamatergic synapses (Fig. 1e) suggests that the decrease in mini frequency is caused by a general defect in the function of presynaptic terminals.

We next examined the properties of spontaneous minis at

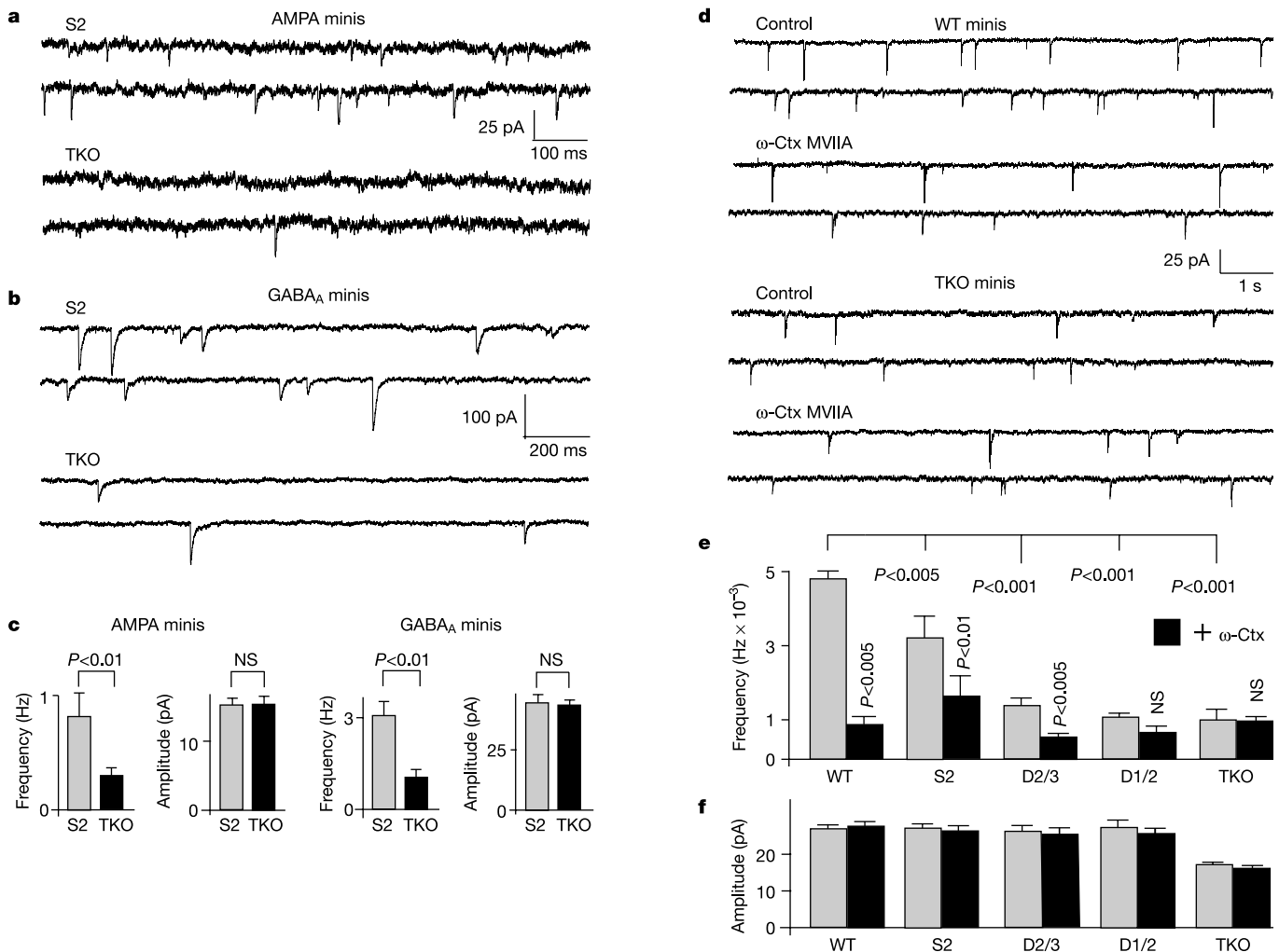


Figure 2 Reduced spontaneous neurotransmitter release (minis) in the neocortex and brainstem from α -neurexin KO mice. **a, b**, Representative recordings of AMPA- and GABA_A-receptor-mediated spontaneous miniature postsynaptic currents (minis) in neocortical slice cultures. Slices were prepared from littermate α -neurexin 2 α KO (control) and triple KO mice. **c**, Frequency and amplitude of minis in neocortical slice cultures (means $\pm$ s.e.m.; AMPA minis: S2, $n = 35$ cells/12 cultures/4 mice, TKO, $n = 35$ cells/9/3; GABA_A minis: S2, $n = 40$ cells/9/3, TKO, $n = 40$ cells/11/2). **d**, Representative recordings of

GABA_A-receptor-mediated minis in acute brainstem slices measured in the pre-Bötzing complex from wild-type and triple KO mice with and without the Ca^{2+} channel blocker ω -conotoxin (0.5 μM MVIIA). **e, f**, Mean frequency (**e**) and amplitude (**f**) of minis in brainstem neurons recorded before and after addition of ω -conotoxin (means $\pm$ s.e.m.; WT, $n = 7$ cells/7 mice; S2, $n = 9/9$; D2/3, $n = 7/7$; D1/2, $n = 5/5$; TKO, $n = 7/7$ mice). In all graphs, statistical significance is indicated above the bars; NS, nonsignificant. **a–c**, neocortex; **d–f**, brainstem.

inhibitory synapses in the pre-Bötzinger complex in acute brainstem slices from various α -neurexin KO mice. Mini frequency in brainstem neurons was also severely depressed by deletion of α -neurexins (Fig. 2d). The decrease in mini frequency correlated with the number of α -neurexins deleted (wild type > α -neurexin 2 α KO > α -neurexin 2 α /3 α double KO > α -neurexin 1 α /2 α double KO > triple KO; Fig. 2e) similar to the relative effect of α -neurexin KO on respiration and survival (Fig. 1b, c). Thus each α -neurexin gene directly contributes to synaptic function. Deletion of α -neurexins had no effect on the mini amplitude except for a decrease in the triple KO (Fig. 2f). Direct application of the GABA_A-receptor agonist muscimol to the postsynaptic neuron elicited similarly robust responses in all genotypes, including the triple KO mice (Supplementary Fig. S8), suggesting that the density of postsynaptic GABA_A receptors was normal. Backfilling of electrophysiologically studied neurons with biocytin showed that reconstructed neurons had a normal appearance in mutant mice, and total capacitance measurements revealed identical cell sizes in wild-type and mutant neurons (Supplementary text).

To determine why α -neurexins are essential for normal spontaneous release in brainstem synapses, we investigated the role of Ca²⁺ channels. Mini frequency was strongly reduced by ω -conotoxin MVIIA (2 μ M; an N-type Ca²⁺ channel blocker)³² in synapses from wild-type mice, suggesting that the activity of N-type Ca²⁺ channels normally contributes to the mini frequency in brainstem synapses. Notably, ω -conotoxin had a much lower effect on mini frequency in

neurexin 2 α KO and neurexin 2 α /3 α double KO mice, and almost no effect on mini frequency in neurexin 1 α /2 α double KO mice and α -neurexin triple KO mice (Fig. 2d, e). ω -conotoxin caused no change in mini amplitudes in any genotype (Fig. 2f). Together these results show that neurotransmitter release is generally impaired in synapses lacking α -neurexins, that the severity of this impairment correlates with the number of mutant genes, and that the impairment is at least partly due to a loss of synaptic Ca²⁺ channel function.

Impaired evoked synaptic transmission in neocortex

We next asked whether the impairment of spontaneous release in α -neurexin KO mice reflects a fundamental dysfunction of synapses that also applies to neurotransmitter release evoked by action potentials. To test this, we first used paired recordings from cultured neocortical slices. We stimulated presynaptic interneurons with trains of four action potentials that were separated by 50 ms. We repeated this stimulus train every 5 s, and recorded synaptic responses in the whole-cell mode (mostly from pyramidal neurons) as GABA_A-receptor dependent postsynaptic currents³³. These experiments revealed that deletion of α -neurexins severely decreased the probability of neurotransmitter release, and additionally impaired release during repeated stimulations (Fig. 3a–e).

Compared with control synapses lacking only neurexin 2 α , neocortical synapses lacking all α -neurexins exhibited an approximately 20-fold reduction in mean amplitude of synaptic responses

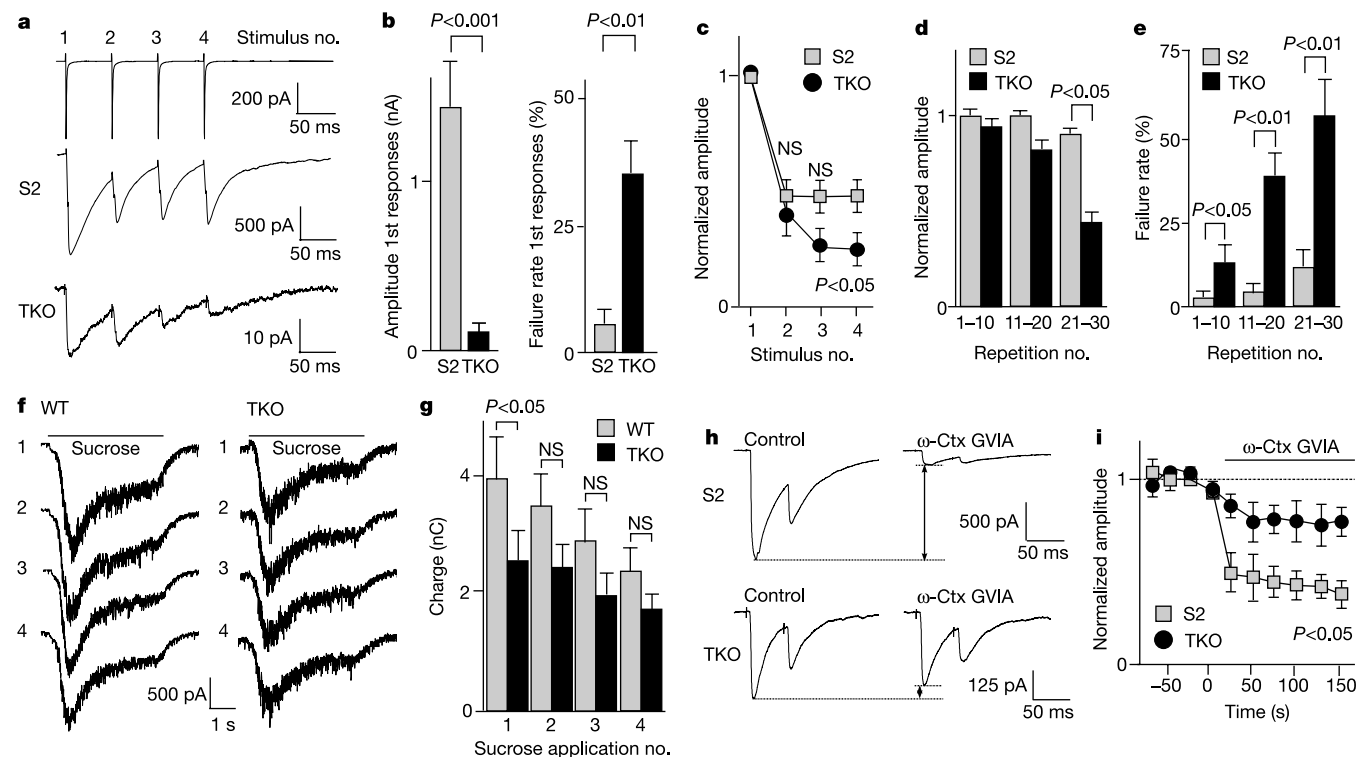


Figure 3 Impaired evoked neurotransmitter release in neocortical synapses from α -neurexin KO mice. **a**, Representative paired recordings of evoked GABA_A-receptor-mediated postsynaptic currents in pyramidal neurons of cultured neocortical slices from littermate neurexin 2 α KO (S2, control) and triple KO mice (TKO). Presynaptic neurons were stimulated by trains of four action potentials separated by 50 ms and repeated every 5 s. **b**, Mean amplitudes and failure rates of synaptic responses to the first action potential in each stimulus train (averaged over 2.5 min). **c**, Mean amplitudes of the second, third and fourth synaptic responses in a stimulus train (normalized to the first response). **d**, **e**, Changes in amplitudes (**d**) and failure rates (**e**) of the first responses over a 2-min stimulation period. **f**, Representative GABAergic synaptic responses elicited by hypertonic

sucrose (0.5 M for 4 s; applications were repeated every 45 s). **g**, Quantification of sucrose responses (plotted as total charge transfer per sucrose application) in wild-type (control) and triple KO mice. **h**, Effect of 2 μ M ω -conotoxin GVIA on GABAergic synaptic responses induced by field stimulation. **i**, Normalized amplitudes of evoked GABAergic synaptic responses as a function of ω -conotoxin application. All panels show means $\pm$ s.e.m.; statistical significance levels are indicated above the diagrams (paired recordings in **a**–**e**: S2, $n = 11$ pairs of cells/4 cultures/2 mice, TKO, $n = 12/7/3$; sucrose stimulation in **f** and **g**: WT, $n = 8$ cells/4 cultures/2 mice, TKO, $n = 6/4/2$; see Supplementary Fig. S9).

(monitored as the first response in each stimulus train), and about a fivefold increase in the mean failure rate (Fig. 3b). Thus deletion of all α -neurexins severely decreased the synaptic release probability. Deletion of α -neurexins also aggravated short-term synaptic depression within individual stimulus trains (Fig. 3c), and increased synaptic depression during multiple stimulus trains (Fig. 3d, e). Within a stimulus train, responses to the second stimulus displayed similar paired-pulse depression in test and control synapses, but responses to the third and fourth stimuli were markedly lower in synapses lacking all α -neurexins than in control synapses (Fig. 3c).

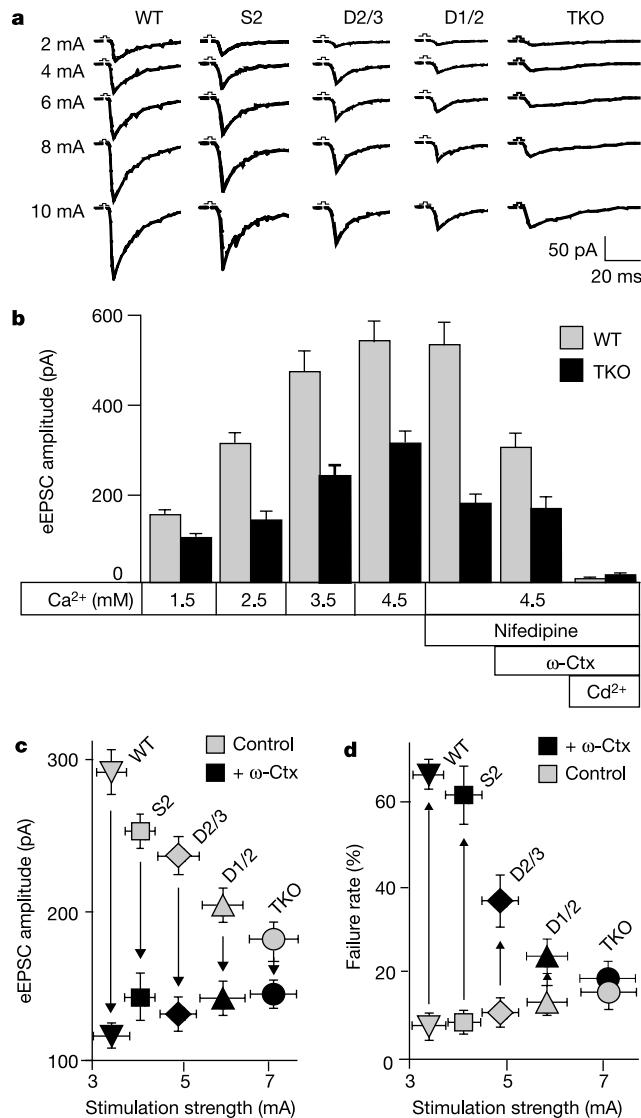


Figure 4 Evoked excitatory synaptic responses in brainstem synapses: effect of ω -conotoxin. **a**, Glutamatergic evoked synaptic responses monitored by whole-cell recordings in hypoglossal motor neurons during field stimulation of axons from the pre-Böttinger complex. **b**, Mean amplitudes of evoked synaptic responses to field stimulations (0.4–5 mA) at increasing extracellular Ca^{2+} concentrations (1.5–4.5 mM Ca^{2+}), with subsequent block of L-type Ca^{2+} channels (10 μM nifedipine), N-type Ca^{2+} channels (0.5 μM MVIIB) and all Ca^{2+} channels (100 μM Cd^{2+}) ($n = 5$ WT and triple KO mice). **c**, **d**, Mean amplitudes (**c**) and failure rates (**d**) of evoked responses in wild-type and various α -neurexin KO mice as a function of stimulation strength in the absence and presence of ω -conotoxin. Stimulation strengths were increased in the α -neurexin KO mice to compensate for the severely impaired release (WT, $n = 5$; S2, $n = 10$; D2/3, $n = 5$; D1/2, $n = 5$; TKO, $n = 6$ mice). All data are means $\pm$ s.e.m.

Furthermore, repeated stimulus trains decreased the amplitudes of synaptic responses in α -neurexin triple KO mice by greater than twofold, and increased their failure rates by greater than twofold. Control synapses, in contrast, exhibited stable synaptic amplitudes and only a small increase in failure rates (Fig. 3d, e).

In principle, the impairment of release by deletion of α -neurexins could be due to a decrease in the number of functionally active synapses (beyond the decrease in GABAergic synapses observed morphologically; see Fig. 1d–f), a disorganization of the secretory apparatus, or a defect in coupling action potentials to exocytosis. To differentiate between these possibilities, we recorded GABA_A-receptor-mediated synaptic responses induced by 0.5 M hypertonic sucrose (Fig. 3f). Hypertonic sucrose and action potentials induce release from the same pool of synaptic vesicles, but hypertonic sucrose directly stimulates the secretory apparatus independent of Ca^{2+} , whereas action potentials trigger release by gating Ca^{2+} influx, which then triggers release^{5,6,34}. Deletion of α -neurexin moderately decreased synaptic responses induced by 0.5 M sucrose (Fig. 3g). Repeated sucrose applications led to a rundown in synaptic responses that was similar in synapses lacking all α -neurexins and in control synapses. The moderate decrease in sucrose-evoked release in synapses from triple KO mice (about 30%) resembled the decrease in the number of symmetric synapses in these mice (about 50%; Fig. 1e), whereas the decrease in evoked responses (about 95%, Fig. 3b) was much more severe. This suggests that the former is due to the loss of GABAergic synapses, whereas the latter reflects impaired triggering of release by action potentials, either because Ca^{2+} influx or Ca^{2+} triggering are altered.

In the brainstem, a loss of N-type Ca^{2+} channel activity seemed to account, at least in part, for the decrease in mini frequency observed in synapses lacking α -neurexins (Fig. 2e). To test whether the massive decrease in inhibitory synaptic transmission in neocortical synapses is also related to impaired Ca^{2+} channel function, we examined the effect of 2 μM ω -conotoxin GVIA, a selective N-type Ca^{2+} channel blocker, on evoked synaptic responses (Fig. 3h). We evoked GABAergic synaptic responses in slice cultures from triple KO mice and neurexin 2 α control KO mice by extracellular stimulation (Fig. 3i). We found that the inhibition caused by ω -conotoxin was much less in the triple KO slice cultures than in the neurexin 2 α KO control slices (neurexin 2 α KO control, $57.5 \pm 6.2\%$ inhibition; triple KO, $26.5 \pm 10.7\%$ inhibition; $P = 0.03$, $n = 6$ cells/6 slices/1 animal; see also Supplementary Fig. S9). Thus at least part of the impairment of synaptic responses derives from a loss of N-type Ca^{2+} channel function.

Impaired evoked synaptic transmission in brainstem

Does the marked reduction in evoked inhibitory responses in neocortical synapses from α -neurexin KO mice reflect a general defect in synaptic responses, as suggested above by the analysis of spontaneous release events (Fig. 2)? To test this question, we examined synaptic transmission in brainstem synapses as a completely different preparation, and measured evoked excitatory synaptic responses in hypoglossal motor neurons using whole-cell patch-clamp recordings (Supplementary Fig. S7). Synaptic responses were elicited by field stimulation of inputs from the pre-Böttinger complex³⁵ with constant currents of 2–10 mA. Even at the highest stimulation strength (10 mA), the amplitudes of excitatory postsynaptic currents were significantly smaller in α -neurexin KO mice than in control mice (Fig. 4a; see also Supplementary Fig. S10). This suggests that similar to inhibitory synaptic transmission in the neocortex, excitatory transmission in the brainstem is impaired. This impairment was not due to a loss of excitatory synapses, as their density in the brainstem was normal (Fig. 1d, f), and was not caused by nonspecific damage to postsynaptic neurons, as the total capacitance of the cells was unchanged (see Supplementary Information).

We then explored the effect of changes in the extracellular Ca^{2+}

concentration on synaptic transmission (Fig. 4b). Stepwise increases in extracellular Ca^{2+} elevated the amplitude of synaptic responses both in wild-type and α -neurexin-deficient neurons. However, at all Ca^{2+} concentrations the responses were lower in α -neurexin-deficient neurons, suggesting that the release abnormality was not due to a shift in the Ca^{2+} affinity of the presynaptic Ca^{2+} sensor. We next measured the relative inhibition of synaptic transmission caused by sequential application of nifedipine (an L-type Ca^{2+} channel blocker), ω -conotoxin MVIIA (an N-type Ca^{2+} channel blocker) and Cd^{2+} (a general Ca^{2+} channel blocker). Nifedipine had no effect on synaptic transmission in wild-type neurons, but depressed synaptic responses in α -neurexin-deficient neurons by approximately 50%. Conversely, ω -conotoxin decreased synaptic responses in wild-type neurons by about 50%, but had little effect in α -neurexin-deficient neurons (Fig. 4b). Cd^{2+} totally blocked synaptic transmission in both wild-type and mutant neurons. These data confirm the conclusion derived from the experiments described above (Figs 2e and 3i) that N-type Ca^{2+} currents are impaired in α -neurexin KO mice, and suggest that

L-type Ca^{2+} currents at least partially compensate for this deficiency.

We next asked whether a loss in Ca^{2+} channel function could explain the phenotype in all α -neurexin KO mice. We probed the effect of ω -conotoxin (0.5 μM MVIIA) on evoked responses mediated by glutamate receptors in brainstem slices (Fig. 4c, d; see also Supplementary Fig. S10). In these experiments, higher stimulation strengths were applied to neurons from α -neurexin KO mice than to controls to compensate for the smaller synaptic responses in the KOs. In spite of the higher stimulus strength, the amplitudes of synaptic responses were still diminished in α -neurexin-deficient neurons (Fig. 4c) and the failure rate was increased (Fig. 4d), which confirms the decrease in release probability observed in neocortical synapses (Fig. 3). The magnitude of the change in evoked release depended on the number of mutant α -neurexins (Fig. 4c, d) in precisely the same order as observed for survival (Fig. 1b), respiratory activity (Fig. 1c) and mini frequency (Fig. 2e); thus each α -neurexin gene contributes to setting synaptic strength.

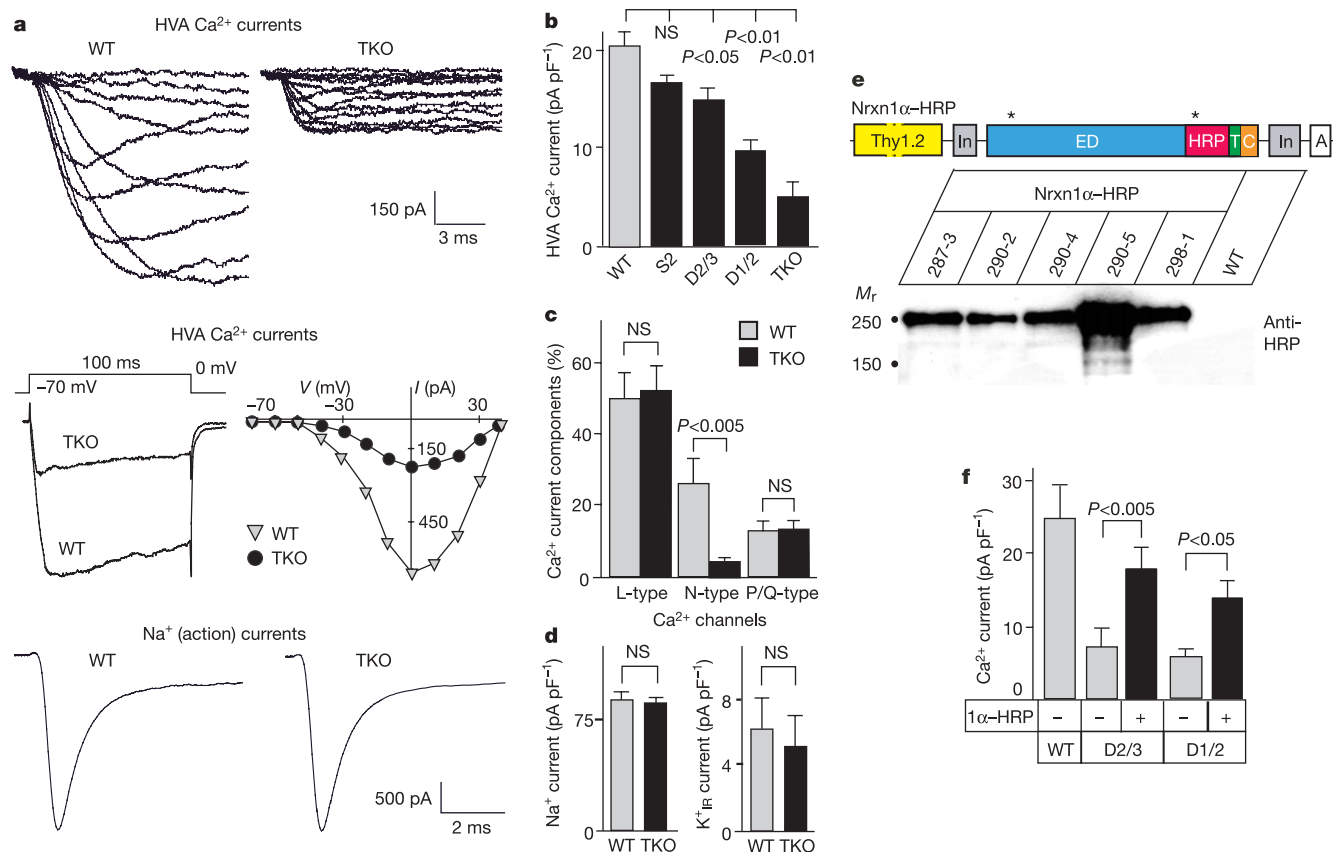


Figure 5 Impaired Ca^{2+} channel function in α -neurexin KO mice: rescue by transgenic α -neurexin 1. **a**, Representative recordings of high-voltage-activated (HVA) Ca^{2+} currents (top and middle panels) and voltage-dependent Na^{+} currents (Na^{+} , bottom panel) measured by whole-cell recordings in the pre-Bötzing complex from wild-type (left panels) and triple KO neurons (right panels). Middle panel, sample traces of Ca^{2+} currents during the maximum voltage-clamp depolarizations (0 mV for 100 ms) (left), and Ca^{2+} current-voltage relations for stepwise depolarizations from -70 to +40 mV (right). **b**, Average peak Ca^{2+} current densities induced by depolarization to 0 mV in brainstem neurons from wild-type and various α -neurexin KO mice (WT, $n = 13$; S2, $n = 5$; D2/3, $n = 9$; D1/2, $n = 9$; TKO, $n = 6$ mice). **c**, Relative contributions of pharmacologically isolated L-, N- and P/Q-type Ca^{2+} currents to peak Ca^{2+} currents in wild-type ($n = 8$) and triple KO ($n = 6$) neurons. Ca^{2+} current densities were measured with sequential additions of 10 μM nifedipine, 0.5 μM ω -conotoxin MVIIA and 0.2 μM agatoxin IVA.

d, Peak Na^{+} (left) and K^{+} current (right) densities in wild-type and α -neurexin triple KO mice (WT, $n = 8$ and TKO, $n = 6$ mice for both Na^{+} and K^{+} currents). **e**, Transgenic expression of α -neurexin 1 as a fusion protein with HRP under control of the Thy1.2 promoter. Top panel shows the transgenic vector. Yellow, Thy1.2 promoter; blue, extracellular domains of α -neurexin 1; red, HRP; green, T = transmembrane region; orange, cytoplasmic tail; grey, intronic sequences; white, polyadenylation sequence. The bottom panel is an immunoblot of multiple transgenic lines using affinity-purified HRP antibodies. **f**, Average Ca^{2+} current densities from neurons in the pre-Bötzing complex from wild-type ($n = 12$ mice) and littermate α -neurexin double KO mice with and without the α -neurexin 1-HRP transgene (D2/3, $n = 8$ mice; D2/3 & Nrnx1 α -HRP, $n = 11$; D1/2, $n = 3$; D1/2 & Nrnx1 α -HRP, $n = 3$). Data shown are means $\pm$ s.e.m.; statistical significance is indicated above the diagrams.

In wild-type brainstem synapses, ω -conotoxin caused an approximately threefold decrease in the amplitude of synaptic responses, and an approximately tenfold increase in failure rate, suggesting that these synapses primarily use N-type Ca^{2+} channels for neurotransmitter release³⁵. In synapses from α -neurexin KO mice, however, ω -conotoxin had smaller effects on the synaptic

amplitudes and failure rates (Fig. 4c, d)—the more α -neurexins that were deleted, the lesser the effects of ω -conotoxin on the amplitudes and failure rates of synaptic responses. In the extreme, α -neurexin triple KO mice were almost insensitive to ω -conotoxin. Thus, similar to the neocortex (Fig. 3h, i), the presynaptic defect in evoked synaptic transmission in the brainstem of α -neurexin KO mice is at least in part due to a loss of Ca^{2+} channel function.

Reduced Ca^{2+} currents

To directly test the function of voltage-dependent Ca^{2+} channels in α -neurexin KO mice, we recorded Ca^{2+} currents from neurons in the pre-Bötzinger area of the brainstem. Voltage clamp limitations make it difficult to record voltage-gated Ca^{2+} currents from neurons *in situ* when these neurons participate in synaptic networks. Such recordings are possible for neurons of the pre-Bötzinger complex because these neurons are relatively small. It seemed to us particularly important to record from neurons in a functional synaptic network because α -neurexins are presynaptic cell-adhesion molecules whose function may be coupled to synapse formation (see below), and may only be apparent in a physiological synaptic network.

Whole-cell Ca^{2+} currents activated by high voltage were severely depressed in triple KO neurons compared with control neurons (Fig. 5a, b). In contrast, voltage-gated sodium (Na^+) and potassium currents (K^+ , IR = inward rectifying) exhibited no significant change (Fig. 5a, d). Comparative analyses of various combinations of α -neurexin KOs showed that the decrease in Ca^{2+} currents directly correlated with the number of deleted α -neurexins (Fig. 5b), identical to the impairment in survival and breathing (Fig. 1b, c), spontaneous release (Fig. 2d) and evoked synaptic transmission (Fig. 4). Current-voltage curves did not suggest major changes in the voltage-dependent gating of Ca^{2+} channels (Fig. 5a), but space-clamp limitations precluded a precise biophysical analysis.

Different types of voltage-gated Ca^{2+} channels are expressed in neurons in developmentally regulated patterns, and are inhibited by distinct pharmacological agents^{32,36,37}. The magnitude of the decrease in voltage-gated Ca^{2+} currents in triple α -neurexin KO mice exceeds that observed in KO mice lacking individual Ca^{2+} channels^{38–41}, suggesting that more than one type of Ca^{2+} channel was affected. To evaluate the contributions of different types of Ca^{2+} channels to the reduction in Ca^{2+} currents, we sequentially blocked L-type channels with 10 μM nifedipine, N-type channels with 0.5 μM ω -conotoxin MVIIA, and P/Q-type channels with 0.2 μM ω -agatoxin IVA (Fig. 5c). R-type Ca^{2+} channels were assessed by blockage of the remaining Ca^{2+} conductances with Cd^{2+} (data not shown). The amplitudes of all types of Ca^{2+} currents were decreased in the triple KO mice compared with wild-type controls. N-type Ca^{2+} channels were the most severely affected and almost completely abolished (Fig. 5c), consistent with the lack of effect of ω -conotoxin on neurotransmitter release (Figs 2d and 4b–d). Although we showed above that L-type Ca^{2+} currents can partially substitute for N-type Ca^{2+} currents in synapses from α -neurexin KO mice (Fig. 4b), the contribution of L-type Ca^{2+} currents to the total Ca^{2+} current was not increased in the triple KO (Fig. 5c), suggesting that the functional compensation of N-type Ca^{2+} channels by L-type Ca^{2+} channels is not due to a major upregulation of L-type Ca^{2+} channels.

Transgenic rescue of Ca^{2+} currents

We next asked whether deletion of α -neurexins directly caused the Ca^{2+} channel phenotype in the α -neurexin KO mice, or whether an unrelated effect may be responsible. To test this, we generated transgenic mice that express α -neurexin 1 α under control of the Thy1.2 promoter (Fig. 5e). Transgenic α -neurexin 1 α was produced as a fusion protein with an internal epitope (horseradish peroxidase (HRP)) to facilitate detection. The transgene was expressed in most of the neurons throughout the brain, including the brainstem

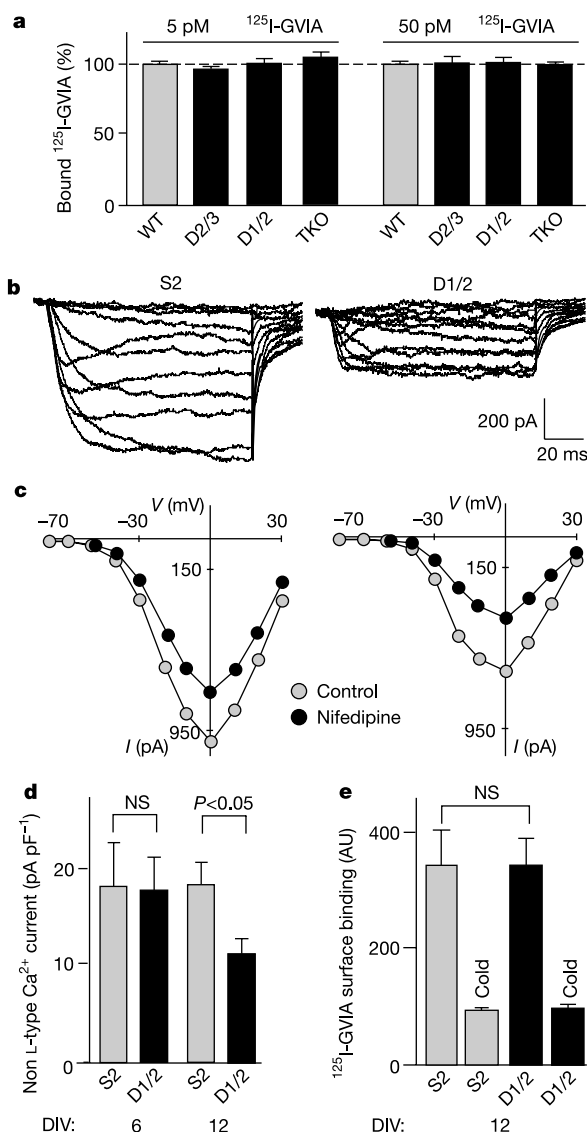


Figure 6 Ca^{2+} channels are normally expressed in α -neurexin KO mice but are partly inactivated after synaptic contacts are established. **a**, Specific binding of ^{125}I -labelled ω -conotoxin GVIA at 5 and 50 pM to brain membranes from wild-type and various α -neurexin KO mice (means $\pm$ s.e.m.; wild type, $n = 4$ mice; D2/3, $n = 3$; D1/2, $n = 3$; TKO, $n = 4$). **b**, **c**, Representative Ca^{2+} current traces (**b**) and Ca^{2+} current-voltage relations before and after addition of 10 μM nifedipine (**c**) in cultured primary hippocampal neurons of littermate α -neurexin 2 α KO control mice (S2) and α -neurexin 1 α /2 α double KO mice (D1/2) after 12 days *in vitro*. **d**, Average peak Ca^{2+} current densities in hippocampal neurons. Currents were recorded after blocking L-type channels with 10 μM nifedipine in pyramidal-like neurons from control and α -neurexin double KO mice cultured for 6 (6 DIV; S2, $n = 6$ cells/6 cultures/3 mice; D1/2, $n = 4/4/3$) and 12 days (12 DIV; S2, $n = 9/9/4$; D1/2, $n = 6/6/3$). **e**, Surface binding of ^{125}I -labelled ω -conotoxin GVIA to live hippocampal neurons cultured for 12 days. 50 pM ^{125}I -GVIA was bound to cultures of identical density derived from individual hippocampi of control (S2, $n = 8$ cultures/4 mice) and α -neurexin double mutant KO mice (D1/2, $n = 4$ cultures/2 mice) in the absence or presence (cold) of excess unlabelled GVIA (30 nM) to monitor nonspecific binding.

(Supplementary Fig. S11). Immunoelectron microscopy revealed that the transgenic neuroligin 1α HRP fusion protein was selectively targeted to presynaptic plasma membranes (A.R., W.Z., R.E.H., T.C.S. and M.M., manuscript in preparation). We crossed the transgenic mice with neuroligin 1α/2α and neuroligin 2α/3α double KO mice, and analysed Ca^{2+} currents in littermate offspring (Fig. 5f). In both double KO mice, the transgene caused a greater than twofold increase in neuronal Ca^{2+} currents, validating a role for α-neurexins in regulating the activity of Ca^{2+} channels. The degree of rescue varied among neurons, possibly because of differences in the expression levels of the transgene (Supplementary Fig. S11).

Impaired synaptic activity of Ca^{2+} channels

To identify a possible cause for the decrease in Ca^{2+} channel function in α-neurexin KO mice, we measured the protein levels of N-type Ca^{2+} channels in brain membranes from wild-type and α-neurexin-deficient mice. Using ^{125}I -labelled ω-conotoxin GVIA, we found that the levels and affinity of N-type Ca^{2+} channels were indistinguishable between wild-type and KO mice lacking various α-neurexins (Fig. 6a; see also Supplementary Fig. S12) in spite of the marked decrease in ω-conotoxin-sensitive Ca^{2+} currents in α-neurexin KO mice (Fig. 5c). Furthermore, quantitative immunoblotting demonstrated that the levels of P/Q-type Ca^{2+} channel proteins were also unchanged (see Supplementary Table S2), confirming that the decrease in Ca^{2+} channel activity was not caused by a loss of Ca^{2+} channel proteins.

As Ca^{2+} channel protein levels were apparently normal, Ca^{2+} channel dysfunction may be due to a deficit in the transport of Ca^{2+} channels to the cell surface and/or inactivation of cell-surface Ca^{2+} channels. Such defects could be related to the formation of synapses because α-neurexins are synaptic cell-adhesion molecules, and a change in Ca^{2+} channels may not be apparent when a neuron fails to form synapses. As a first step towards testing this hypothesis, we cultured hippocampal neurons from double KO mice lacking neuroligin 1α and 2α and, as a control, single KO mice lacking only neuroligin 2α. We then measured Ca^{2+} currents in the cultured neurons after 6 and 12 days *in vitro* (Fig. 6b); time points before and after functional synapses are established⁴². Although voltage-clamp conditions were suboptimal, Ca^{2+} currents with apparently normal current–voltage relationships could be recorded (Fig. 6c). When we quantified the steady-state amplitude of Ca^{2+} currents activated by depolarization from -70 mV to 0 mV , we observed no difference between the neuroligin-2α- and neuroligin-1α/2α-deficient neurons after 6 days *in vitro*. However, after 12 days *in vitro*, the neuroligin-1α/2α-deficient neurons exhibited a nearly twofold decrease in non L-type, nifedipine-insensitive Ca^{2+} currents compared with neuroligin-2α-deficient neurons (Fig. 6d, e). Thus Ca^{2+} channel activity as such does not require α-neurexins, but becomes dependent on α-neurexins at the time of synaptogenesis. We then tested whether the decrease in Ca^{2+} channel function was due to a loss of surface expression of Ca^{2+} channels. The amount of surface binding of ^{125}I -labelled ω-conotoxin GVIA was indistinguishable between neuroligin 2α control neurons and neuroligin 1α/2α double KO neurons (Fig. 6e), suggesting that cell surface expression of Ca^{2+} channels was normal.

The neuroligin hypothesis

Marked advances have been made recently in the understanding of postsynaptic mechanisms, for example the discovery of stargazins and the molecular description of glutamate receptor recycling^{43,44}. Furthermore, neuroligins and SynCAMs have been identified as postsynaptic molecules that can induce differentiation of presynaptic nerve terminals^{16,45}. However, little is known about the presynaptic mechanisms that assemble the secretory machinery for neurotransmitter release in nerve terminals precisely opposite to the postsynaptic density, and that control the activity and location of Ca^{2+} channels. In the present study, we show that

α-neurexins, presynaptic cell-adhesion molecules that are coupled to intracellular PDZ domain proteins²², are required for normal neurotransmitter release, and that deletion of α-neurexins impairs the function of synaptic Ca^{2+} channels. Our results reveal a link between two previously unconnected processes—synaptic cell adhesion and voltage-gated Ca^{2+} -signalling—and suggest the hypothesis that α-neurexins couple synaptic cell adhesion to presynaptic Ca^{2+} -channels and other parts of the presynaptic secretory machinery.

Deletion of α-neurexins impaired both spontaneous and evoked neurotransmitter release as measured in excitatory and inhibitory synapses in the brainstem and neocortex. The decrease in mini frequency, the decrease in evoked responses, the increase in failure rates, and the lack of noticeable changes in postsynaptic receptor activity demonstrate a primary presynaptic impairment. This impairment was not due to developmental abnormalities. The only structural change found in α-neurexin KO mice was a selective decline in the number of inhibitory synapses that could not account for the much larger decrease in inhibitory synaptic transmission. This decline could be due to the special role of GABAergic synaptic transmission in driving early development^{28,29}, which may lead to enhanced activity-dependent elimination of inhibitory synapses in α-neurexin KO mice.

A cause for impaired neurotransmitter release in α-neurexin KO mice was found in the decrease in presynaptic Ca^{2+} currents, especially N-type Ca^{2+} currents, as shown by the altered sensitivity of release to Ca^{2+} channel blockers, and confirmed by the decrease in whole-cell Ca^{2+} currents. This was not an indirect consequence of the genetic manipulations because the Ca^{2+} currents could be partially rescued with a neuroligin 1α transgene. Mouse mutants of neuronal Ca^{2+} channels generally have a milder phenotype than the α-neurexin KO phenotype described here, apparently because deletion of a particular Ca^{2+} channel results in upregulation of other Ca^{2+} currents^{38–41}, whereas the α-neurexin KOs affect all types of Ca^{2+} currents. The decrease in N-type Ca^{2+} currents was not due to a loss or impaired cell-surface expression of Ca^{2+} channels as the amounts and affinity of ω-conotoxin binding sites were unchanged, and the level of cell-surface conotoxin binding in hippocampal neurons was indistinguishable between mutants and controls. The presynaptic defects caused by the KOs of α-neurexins are probably not restricted to Ca^{2+} currents, because in neocortical synapses, spontaneous release (minis) is usually Ca^{2+} -independent but was still decreased in the KOs, and because α-neurexin-deficient synapses exhibited an increased depression. This suggests that α-neurexins may be required for the organization of other presynaptic functions as well.

We propose that α-neurexins function as presynaptic cell adhesion and/or cell recognition molecules that couple extracellular synaptic interactions to the intracellular organization of the presynaptic secretory apparatus and Ca^{2+} channels. α-Neurexins are clearly not subunits of Ca^{2+} channels because the function of Ca^{2+} channels is normal in many cells lacking α-neurexins (such as chromaffin or muscle cells). α-Neurexins are not ‘chaperones’ for Ca^{2+} channels because at least N-type channels (which contributed predominantly to the Ca^{2+} current reduction) were transported to the cell-surface in α-neurexin KO mice and exhibited a normal ω-conotoxin affinity. One model to explain how α-neurexins determine the synaptic activity of Ca^{2+} channels is that Ca^{2+} channel activity is negatively regulated in mature neurons, and that synaptic α-neurexins function to selectively empower Ca^{2+} channels at the synapse. Such a mechanism would concentrate Ca^{2+} influx to synapses, and imply a neuroligin-dependent pathway by which postsynaptic neurons could control presynaptic Ca^{2+} channel activity⁸. According to this proposal, α-neurexins act as trans-synaptic cell-adhesion molecules that participate in a modular organization of presynaptic terminals by mediating the localized activation of Ca^{2+} channels. □

Methods

Generation of α -neurexin KO and transgenic mice

α -Neurexin KO mice were generated by deleting the large first exons of the murine α -neurexin genes²³, maintained on a mixed SV129/C57bl6 background, and genotyped by polymerase chain reaction^{13,46} (see Supplementary text and Fig. S1 for the structure of the KO vectors and the breeding and analysis strategies). Respiratory activity was measured by whole-body plethysmography. Transgenic mice were produced as described⁴⁷ using a chimaeric HRP-neurexin 1 α vector encoding neurexin 1 α with an inserted HRP tag as an epitope (A.R., W.Z., R.E.H., T.C.S. and M.M., manuscript in preparation), and analysed as heterozygous transgenic mice on wild-type or α -neurexin KO backgrounds.

Biochemical procedures

SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting, including quantifications of protein levels using ¹²⁵I-labelled secondary antibodies and phosphorimager detection, were carried out as described⁴⁵. Radio-ligand binding studies were performed with membrane fractions (P2) from newborn mice incubated with the indicated concentrations of ¹²⁵I-labelled ω -conotoxin GVIA (NEN) with or without excess cold GVIA (Alamone Labs, concentration 500 $\times$ dissociation constant).

Morphology and image analysis

Brains from newborn mice were immersion-fixed and from adult mice perfusion-fixed and analysed by light and electron microscopy essentially as described⁴⁶ (see Supplementary Information for details).

Neocortical slice culture and electrophysiology

Slices were cultured 25–40 days *in vitro*³⁰ and analysed by whole-cell voltage clamp recordings essentially as described³³ (see Supplementary Information for details). Minis were recorded in 1 μ M tetrodotoxin with 10 μ M 6,7-dinitroquinoxaline-2,3-dione (DNQX; GABA_A-dependent minis) or 0.1 mM picrotoxin (AMPA-dependent minis). In paired recordings, presynaptic action potentials were elicited by 30 trains of four depolarizations to +10 mV (0.5–1 ms) at 20 Hz with 5-s intervals. Hypertonic ACSF containing 0.5 M sucrose, 50 μ M D(-)-2-amino-5-phosphonopivalic acid (AP5), 10 μ M DNQX and 1 μ M tetrodotoxin was pressure-applied (about 400 mm Hg) to the cell soma with a patch pipette (2 μ m diameter) for 4 s with 45-s intervals.

Electrophysiological analysis of brainstem neurons

Analyses were performed essentially as described^{48,49} by whole-cell recordings in acute slices containing the pre-Bötzing complex and hypoglossal motor nucleus from newborn (P1) littermate mice (see Supplementary text and Fig. S7 for details). Cells with a serial resistance of >20 M Ω , a membrane resistance of <0.8 G Ω , or leak currents of >150 pA were excluded. All Ca²⁺, Na⁺ and K⁺ current measurements were corrected using the 'P/4 protocol' that subtracts leak currents measured during four leak-subtraction pre-pulses applied immediately before each voltage step⁵⁰. Minis were recorded at a Cl[−] reversal potential of about 0 mV in 10 μ M CNQX; signals with amplitudes of at least 2.5 times above background noise were selected, and statistical significance was tested in each experiment. Evoked responses were monitored in the presence of 2 μ M strychnine and 2 μ M bicuculline in patched hypoglossal motor neurons in response to 0.1 Hz field stimulation of axons of the pre-Bötzing complex. Peak amplitudes were averaged from 25 consecutive excitatory postsynaptic currents. Input resistance was recorded before every 5th stimulus by a −10 mV voltage step (20 ms) from a holding potential of −70 mV. Measurements of Na⁺ and inwardly rectified K⁺ current were made in the presence of 0.3 mM Cd²⁺ as described in Supplementary Information. Voltage-activated Ca²⁺ currents were measured with patch electrodes containing (in mM): 110 CsCl₂, 30 TEA-Cl, 1 CaCl₂, 10 EGTA, 2 MgCl₂, 4 Na₃ATP, 0.5 Na₃GTP, 10 HEPES-CsOH, pH 7.3, and with 0.5 μ M tetrodotoxin in the bath. Currents were recorded in response to voltage-step changes from the −70 mV holding potential to test potentials between −80 to +40 mV, and quantified as average peak currents in response to voltage steps from −70 to 0 mV before or after application of nifedipine, ω -conotoxin, ω -agatoxin and Cd²⁺. Voltage-activated Ca²⁺ currents in cultured hippocampal neurons were measured similarly.

Hippocampal culture analysis

Primary neuronal cultures were plated at low density (8,000 cells cm^{−2}) from individual hippocampi of newborn α -neurexin mutant and littermate control mice according to standard procedures⁴². Neurons were isolated in Hanks-HEPES buffer, and maintained in neurobasal medium NB-A (Invitrogen) supplemented with 2% B-27 (Invitrogen). For labelling with ¹²⁵I-labelled ω -conotoxin GVIA (NEN) with or without addition of unlabelled GVIA, intact cultures were washed briefly with Hanks-HEPES buffer, incubated for 10 min with toxin, washed twice, and coverslips exposed on phosphorimager plates for about 2 h. Electrophysiological recordings on hippocampal neurons on 6 or 12 days *in vitro* (DIV) were performed as described above.

Additional methods

All data shown are means $\pm$ s.e.m. Statistical significance was tested with a two-tailed Student's *t*-test except for the morphological data, which were tested in Excel spreadsheets or with Prism software (GraphPad).

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Structure and gating mechanism of the acetylcholine receptor pore

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The nicotinic acetylcholine receptor controls electrical signalling between nerve and muscle cells by opening and closing a gated, membrane-spanning pore. Here we present an atomic model of the closed pore, obtained by electron microscopy of crystalline postsynaptic membranes. The pore is shaped by an inner ring of 5 α -helices, which curve radially to create a tapering path for the ions, and an outer ring of 15 α -helices, which coil around each other and shield the inner ring from the lipids. The gate is a constricting hydrophobic girdle at the middle of the lipid bilayer, formed by weak interactions between neighbouring inner helices. When acetylcholine enters the ligand-binding domain, it triggers rotations of the protein chains on opposite sides of the entrance to the pore. These rotations are communicated through the inner helices, and open the pore by breaking the girdle apart.

The propagation of electrical signals between nerve cells and their targets takes place at the chemical synapse through the action of transmitter-gated ion channels. These fast-acting molecular switches are oligomeric proteins composed of two main functional parts: an extracellular, ligand-binding domain, and a gated, membrane-spanning pore. Neurotransmitter released from the nerve terminal enters the ligand-binding domain on the surface of the target cell, and triggers a transient conformational change that opens the gate in the membrane-spanning pore. Ions then flow selectively through the pore down their electrochemical gradients, giving rise to a change in membrane potential.

The acetylcholine (ACh) receptor, at the nerve–muscle synapse, is a member of a superfamily of transmitter-gated ion channels, which includes the serotonin 5-HT₃, γ -aminobutyric-acid (GABA_A and GABA_C) and glycine receptors¹. It has a cation-selective pore, delineated by a ring of five subunits (α , α , β , γ or ϵ , δ), that opens upon binding of ACh to distant sites in the two α -subunits at or near the subunit interfaces^{2–4}. There are four predicted membrane-spanning segments, M1–M4, in each subunit. The second membrane-spanning segment, M2, shapes the lumen of the pore, and forms the gate of the closed channel. Although much information has been obtained about the roles of individual amino acids in affecting ion transport, and about their relative positions on the membrane-spanning segments, their detailed three-dimensional arrangement has not yet been visualized in this receptor, or in any other transmitter-gated ion channel. Nor is it known how the ACh-triggered conformational change is communicated through the membrane to open the pore.

The (muscle-derived) electric organ of the *Torpedo* electric ray is highly enriched in ACh-receptor-containing membranes, and has been a valuable source of tissue for physiological and biochemical studies of neurotransmission for more than 50 years. The isolated postsynaptic membranes are also amenable to structure analysis by electron microscopy. They convert readily into tubular crystals, having receptors and intervening lipid molecules organized like they are *in vivo*^{5,6} (Fig. 1), and enable different functional conformations to be investigated under near-physiological ionic conditions. A description of the amino-terminal ligand-binding domain of the receptor has been obtained by fitting the β -sheet core structure from a homologous pentameric ACh-binding protein, AChBP⁷, to the three-dimensional densities determined from electron images⁸. However, the quality of these images and distortions of the crystal lattice limited previous descriptions of the pore, revealing some

α -helical folding, but only in the pore-lining segments⁹. By recording exceptional images at liquid-helium temperatures¹⁰ and applying a computational method to correct for the distortions¹¹, we have now extended the resolution to 4 Å. We report here an atomic model of the pore domain based on the 4-Å density map, and propose an explanation of how the pore opens when ACh binds to the receptor.

Structure determination

Tubular crystals, having helical symmetry, were grown from *Torpedo marmorata* membranes, and imaged in thin films of amorphous ice (see Methods). Altogether 359 images ($\sim 10^6$ receptors), involving four helical families, were analysed.

The tubes were too small to yield accurate amplitudes by electron diffraction, so the amplitude, as well as the phase terms, had to be measured from Fourier transforms of the images, and then added vectorially to enhance the signal-to-noise ratio. The amplitudes showed resolution-dependent fading, which was compensated by scaling the measured values against values calculated from a model



Figure 1 Cross-section of a tubular crystal, at low resolution. The receptor protein projects from either side of the membrane, visible as two concentric rings of density, 30 Å apart. A single receptor, cut centrally, is shown at the top. The membrane-spanning pore and the N-terminal ligand-binding domain, shaping a large central vestibule, are outlined by red and green rectangles, respectively. The surfaces encompassing the hydrophobic core of the membrane are assumed to lie along the centres of the rings of density⁴⁸.

Table 1 Image data

	Helical family				Combined
	(−16,6)	(−17,5)	(−15,7)	(−18,6)	
Tube diameter (Å)	770	760	788	832	
Number of images	160	77	72	50	359
Number of receptors	4.8×10^5	2.2×10^5	2.3×10^5	1.4×10^5	1.1×10^6
Mean defocus (μm; ±s.d.)	1.28 ± 0.29	1.27 ± 0.17	1.32 ± 0.19	1.32 ± 0.25	
Segment length (Å; ±s.d.)	683 ± 52	685 ± 54	676 ± 53	674 ± 49	
Number of layer-lines	1,092	1,065	1,057	1,041	
Independent Fourier terms	1.12×10^5	1.08×10^5	1.04×10^5	1.06×10^5	
Phase errors (degrees)*					
> 9.5 Å	4.9	7.5	6.9	8.4	3.3
9.5–5.5 Å	22.1	35.9	37.0	39.1	13.8
5.5–4.3 Å	47.9	63.8	64.3	68.4	33.2
4.3–4.0 Å	56.5	71.8	71.3	77.2	41.6

*Amplitude-weighted phase differences between independent half-data sets in successive resolution zones; random value, 90°.

based on the structure of AChBP (see Methods). The phases were significant to 4-Å resolution after the terms from many images of the same helical family had been added (Table 1). Structures were synthesized from the amplitude and phase terms in each family and combined, by averaging in real space, to obtain the final three-dimensional density map.

The real-space averaging brought about a substantial improvement in signal/noise ratio (Fourier shell correlation coefficient of 0.5 (refs 12, 13) at 4.0 Å; Supplementary Fig. 1), and enabled the polypeptide chains to be fitted to the densities (see Methods) after assigning the four helical segments as in ref. 8, with the beginning of M1 aligning with the carboxy-terminus of the corresponding portion in the ligand-binding domain. Figure 2 gives examples of the chains superimposed on the densities.

Interface with ligand-binding domain

Experiments with chimaeric molecules first showed that separate stretches of the amino-acid sequence are used to make the ligand-binding region and the membrane-spanning pore of ion channels in the ACh receptor superfamily¹⁴. In the structure, the interface between these two parts is marked by an abrupt transition from a

predominantly β-sheet fold to an α-helical fold (Fig. 3a). Surprisingly, we find that this interface is not at the surface of the 30-Å-thick membrane (indicated in Fig. 1), but about 10 Å away. Thus the domain shaping the lumen of the pore is longer than is commonly assumed, with the α-helical segments, M1–M4, each continuing for some distance beyond the hydrophobic core of the membrane on the extracellular side.

Pentameric pore

The pore presents a tapering pathway for the ions when viewed from the synaptic cleft, and is encircled by a near-perfect five-fold arrangement of α-helical segments, in which each subunit resembles the blade of a propeller (Fig. 3b). The inner, pore-lining helices (M2) tilt radially inwards toward the central axis until they reach the middle of the membrane. In contrast, the outer helices (M1, M3 and M4) tilt both radially toward, and tangentially around, the central axis. This gives rise to a left-handed coiling, with M1 and M3 twisting together in a notably regular way (see also Supplementary Information movie).

The helices are splayed apart on the extracellular side of the membrane (centre-to-centre separation: 12.5–15 Å; Fig. 2a), and

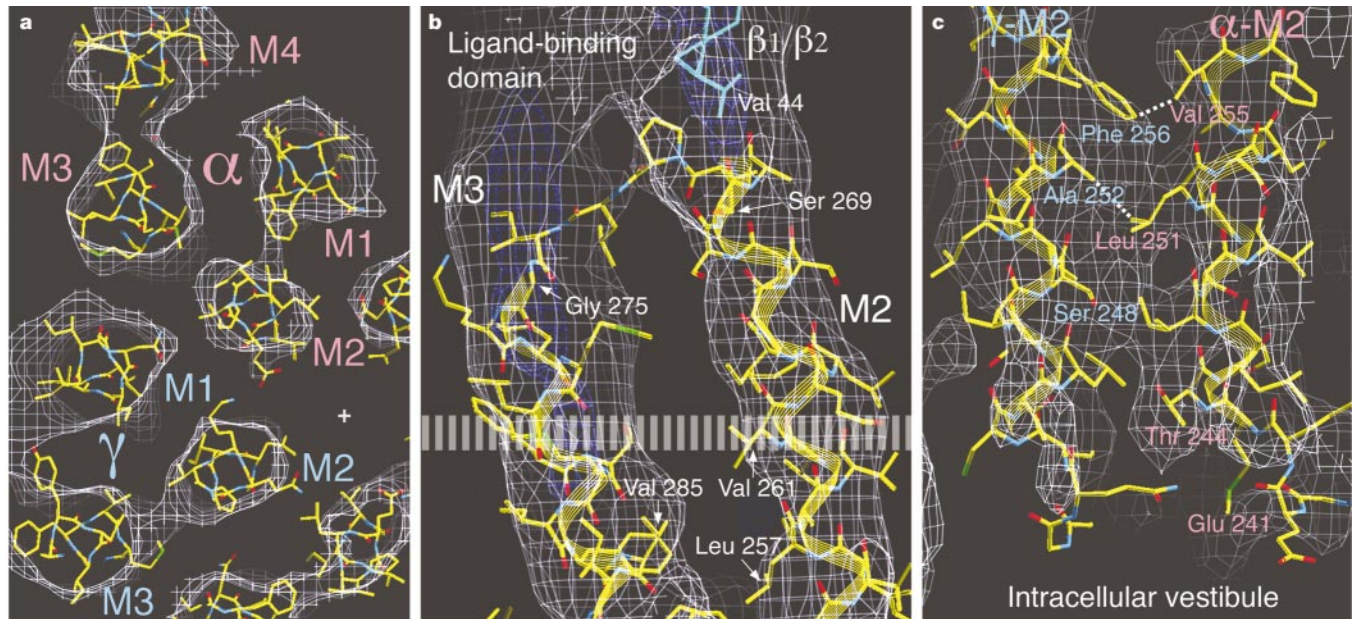


Figure 2 Representative portions of the polypeptide chains superimposed on the density map. **a**, Cross-section through the α-helices at the outer membrane surface; the cross denotes the pore axis; contours at 1.5σ. **b**, Longitudinal section through the M2, M3 helices, and the aligned β1/β2 loop (α-subunit); the broken line denotes the membrane

surface; contours at 2σ (white) and 4σ (blue). **c**, Interaction between the α and γ M2 helices, viewed from the pore axis; putative hydrophobic contacts involving α-Leu 251 and α-Val 255 are indicated by dotted lines; the M2 helices end abruptly near α-Thr 244; contours at 2σ.

may possibly allow ions at the membrane surface to enter the pore laterally, bypassing the alternative route through the outer vestibule (Fig. 1). The largest openings are at the subunit interfaces, and extend into the ligand-binding domain (asterisk, Fig. 3a).

The sets of inner and of outer helices come together separately near the middle of the membrane, creating intervening spaces having dimensions comparable with those of the central hole (Fig. 3c). This suggests that there are just two essential structural components of the pore: an inner ring (blue) that interacts directly with the centrally passing ions, and an outer shell, or scaffold (red), that sequesters this ring away from the surrounding lipids. Such partitioning would be consistent with the findings of earlier experiments on the tubular crystals, which showed that the inner part moves when the receptor is activated, whereas the outer part remains unchanged¹⁵.

It is notable that glycine and GABA_A receptors, in the ACh receptor superfamily, have specific binding sites for alcohols and anaesthetics thought to be in water-filled cavities behind the inner (M2) helices¹⁶. The implicated amino acids are Ser 267 (glycine receptor) and Ser 270 (GABA_A receptor) on the M2 segments of the α -subunits¹⁷. These residues align with α -Leu 257 (arrowhead, Fig. 3c), which faces the space between the inner and the outer sets of helices. Hence this space must be where the alcohols/ anaesthetics bind in the other receptors.

Subunit topology and folding

Each subunit of the pore corresponds to a single protein domain, with maximum dimensions of ~ 50 Å both normal to and parallel to

the membrane plane. The domain consists of the four membrane-spanning helices, M1–M4, predicted by hydropathy plots, the extensions of these helices beyond the outer membrane surface, and connecting loops (Fig. 4a). The ends of the transmembrane portions of M1, M2 and M4 are framed by polar and/or negatively charged groups (at: α -Asn 217 and α -Asp 238; α -Glu 241 and α -Glu 262; α -Asp 407 and α -Gly 428).

The subunit fold is like that of the 'classical' 4- α -helical bundle found in myohemerythrin and tobacco mosaic virus, with the helices next to each other in the sequence being arranged in consecutive positions around the bundle (Fig. 4b). The group of helices, M1, M3 and M4, appears to be stabilized in the membrane by clustering of hydrophobic side chains around a central aromatic residue (α -Phe 233). Helix M2 makes no extensive van der Waals contacts with either M1 or M3, suggesting that it is mainly separated from these helices by water-filled space. There are, however, several close appositions between hydrophobic side chains on M2 and M3 (for example, Leu 250-Ile 296; Leu 257-Ile 289, and Val 261-Val 285 on the α -subunits) and on M2 and M1 (Leu 253-Phe 225). These (potential) interactions are likely to affect relative movements of the inner helices and outer shell of the pentamer during gating.

When individual subunits are aligned by bringing them into strict five-fold register, the helices M1, M2 and M3 superimpose well, especially over their membrane-spanning portions (r.m.s. variations in C α positions: 0.53 Å (M2); 0.82 Å (M1); 1.04 Å (M3)). Helix M4 is less precisely positioned (r.m.s. variation: 1.74 Å), and comes away from the others, by variable amounts, at its extracellular end.

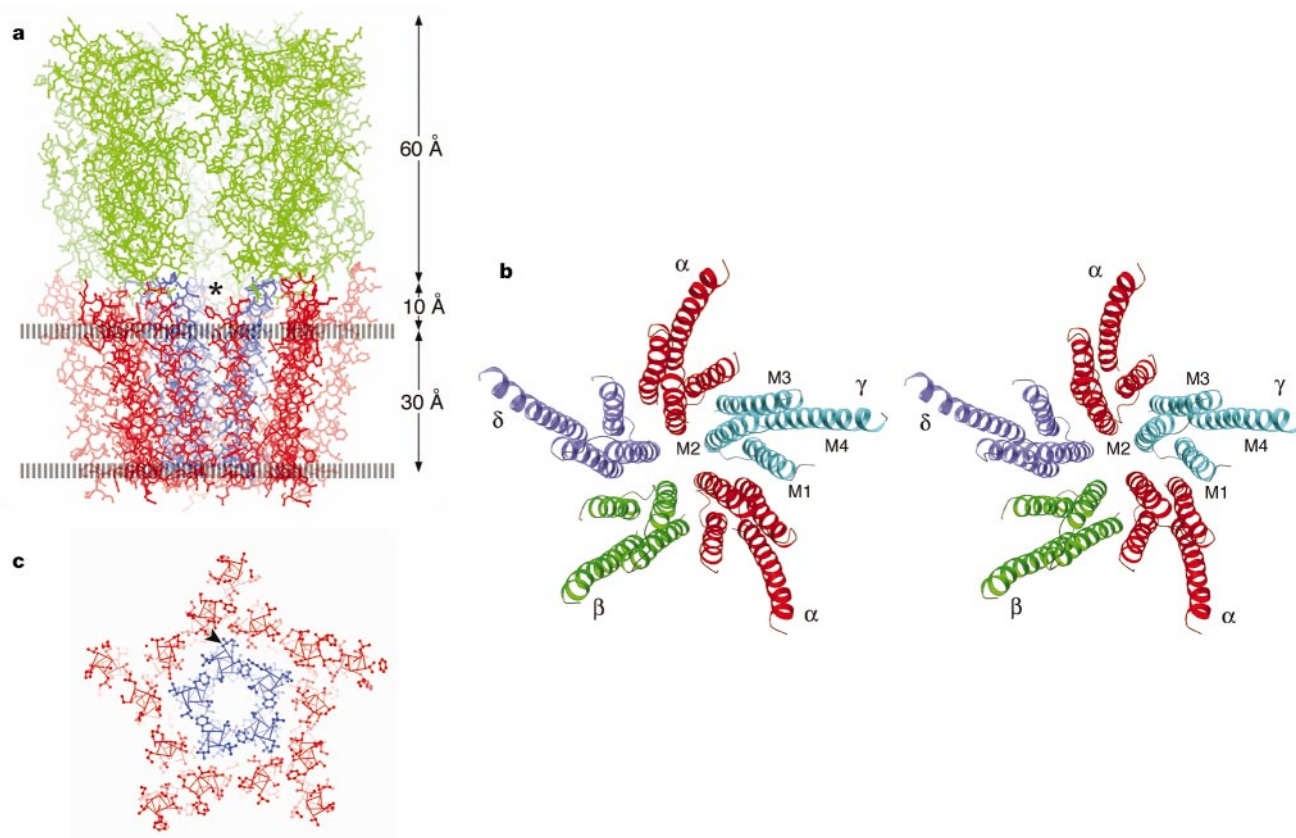


Figure 3 Pentameric structure of the pore. **a**, View normal to the receptor axis showing the α -helical pore structure (blue, pore-facing; red, lipid-facing helices) in relation to the membrane surfaces (broken lines) and the β -sheet structure (green) comprising the ligand-binding domain⁸ (ball-and-stick representation); the asterisk denotes open space at a subunit interface. **b**, Stereo view of the pore, as seen from the synaptic cleft, with

subunits shown in different colours (α , red; β , green; γ , cyan; δ , blue)⁸. **c**, Cross-sectional slab through the pentamer at the middle of the membrane, showing partitioning of the structure into pore- and lipid-facing parts, with intervening spaces (ball-and-stick representation); the arrowhead identifies α -Leu 257 (see text).

The division of the structure into lipid- and water-facing surfaces is in good agreement with the results of experiments in which specific residues have been labelled using either hydrophobic or hydrophilic probes. Several of the residues on M1, M3 and M4 facing the interior of the membrane have been labelled with lipophilic reagents that can be photoactivated^{18,19} (red regions; Fig. 4c). Others that face the lumen of the pore, or are outside the membrane interior, have been identified by cysteine substitution and labelling with small water-soluble compounds^{20,21} (blue regions; Fig. 4d). In addition, several of the identified water-accessible residues distributed over M2 lie between this helix and the others (α -Leu 250; α -Leu 258; the residues on the β -subunit aligning with α -257 and α -261 in Fig. 4a), consistent with the finding that M2 makes minimal contact with the outer shell of the pentamer (Figs 2b and 3c). Not surprisingly, some of the water-accessible residues on M2, and one on M1 (α -Leu 228)¹⁸, have also been labelled by small hydrophobic reagents (green regions; Fig. 4d).

Ion conduction path

The M2 helices shaping the central conduction path are 40-Å long (including the portions outside the membrane), and are slightly kinked at α -Pro 265 and in the vicinity of α -Leu 251 (Fig. 5a). These helices traverse the membrane in register with sets of homologous residues at each level forming rings of chemically distinct environments facing the lumen of the pore^{22,23} (Fig. 5b). The rings are predominantly non-polar, and would present a relatively inert surface to diffusing ions, but two of them (the rings at α -Ser 266 and at α -Glu 262) contain negatively charged groups, which would be expected to influence transport when the pore is open by raising the local cation concentration while lowering the concentration of anions. Mutation combined with electrophysiological experiments had drawn attention to the likely importance of these rings in affecting cation transport^{24,25}. A third negatively charged ring shown to influence ion conduction, the 'intermediate ring' at α -Glu 241 (ref. 25), is in the loop region slightly beyond the ends of the M2 helices, and framing the intracellular entrance of the pore. Nearby

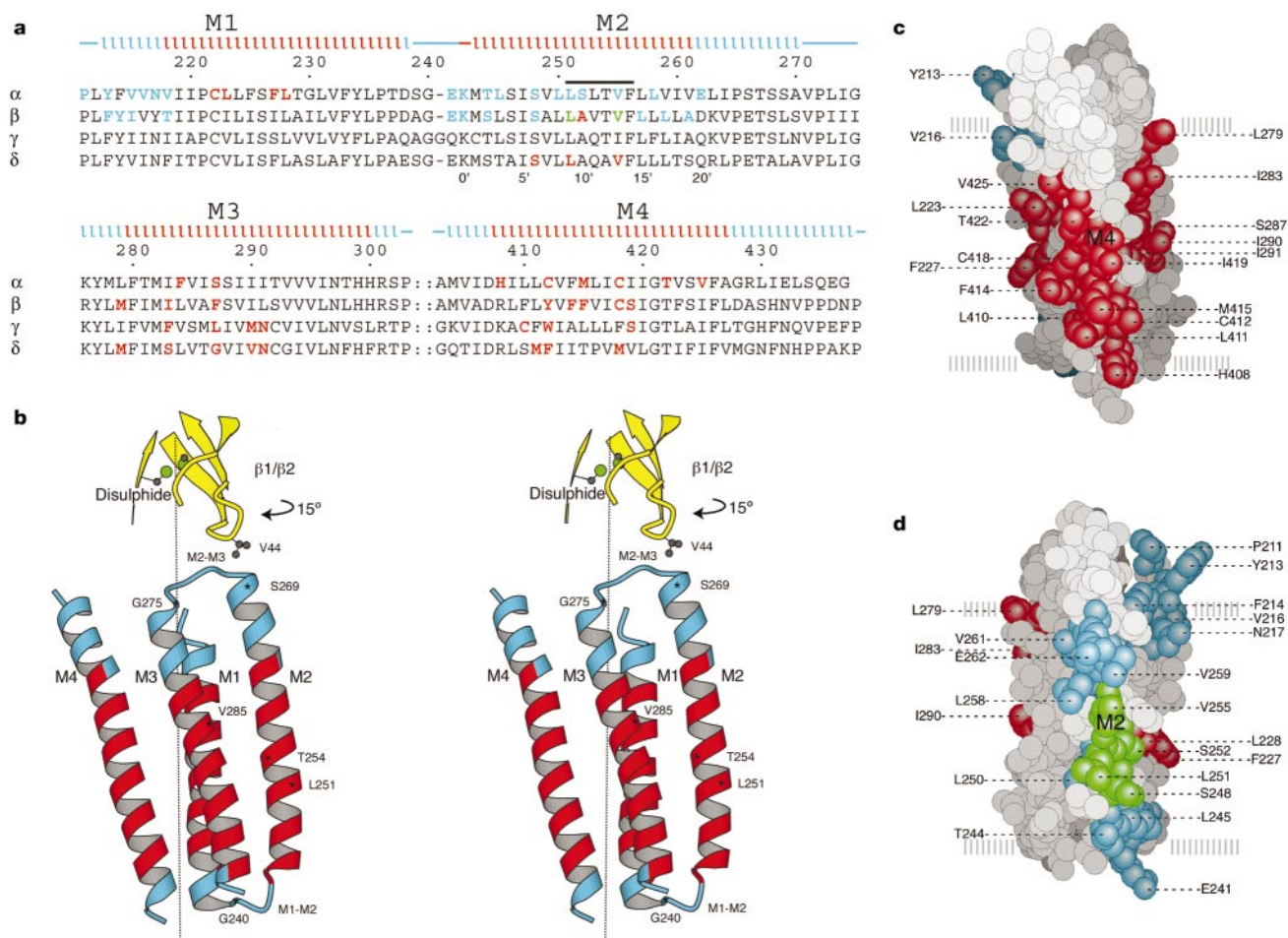


Figure 4 Overview of a pore-forming subunit. **a**, Amino-acid sequences (*Torpedo californica*) of the pore domain of the four aligned polypeptides (α -subunit numbering). The positions of the helical segments, M1–M4, and connecting loops (continuous lines) are indicated above the sequence (red, inside membrane; blue, outside membrane). The gate region on M2 is indicated by the black bar; residues along M2 are also identified according to the primed numbering system²⁶ (thus 9' corresponds to α -Leu 251). Highlighted residues have been labelled with hydrophobic probes^{18,19} (red), with hydrophilic methanethiosulphonate derivatives after substitution by cysteine^{20,21} (blue), or with both (green). The extended loop between M3 and M4 is not shown. **b**, Stereo view of the subunit fold (α -subunit), as seen from the subunit interface, with portions inside and outside the membrane coloured respectively red and blue. The M2–M3 loop (α -Val

271– α -Gly 275) is resolved in all subunits; the M1–M2 loop (α -Ser 239– α -Lys 242) is less well defined, and the trace shown represents the most likely assignment. Also shown are the locations of the inner-sheet loop, β 1/ β 2, and the 'cys-loop' disulphide bridge from the ligand-binding domain⁸. α -Val 44 in the β 1/ β 2 loop docks into the hydrophobic pocket at the end of M2 (see Fig. 2b). The arrow denotes the 15° rotation of the β 1/ β 2 loop about an axis through the disulphide bridge (vertical line) which leads to opening of the pore. **c**, **d**, Space-filling representations of the α -subunit, viewed from outside (**c**) and inside (**d**) the pore, showing the locations of the residues highlighted in **a** relative to the membrane surfaces (broken lines); residues in red, blue and green are labelled with hydrophobic probes, with hydrophilic probes, and with both, respectively (information from all subunits combined).

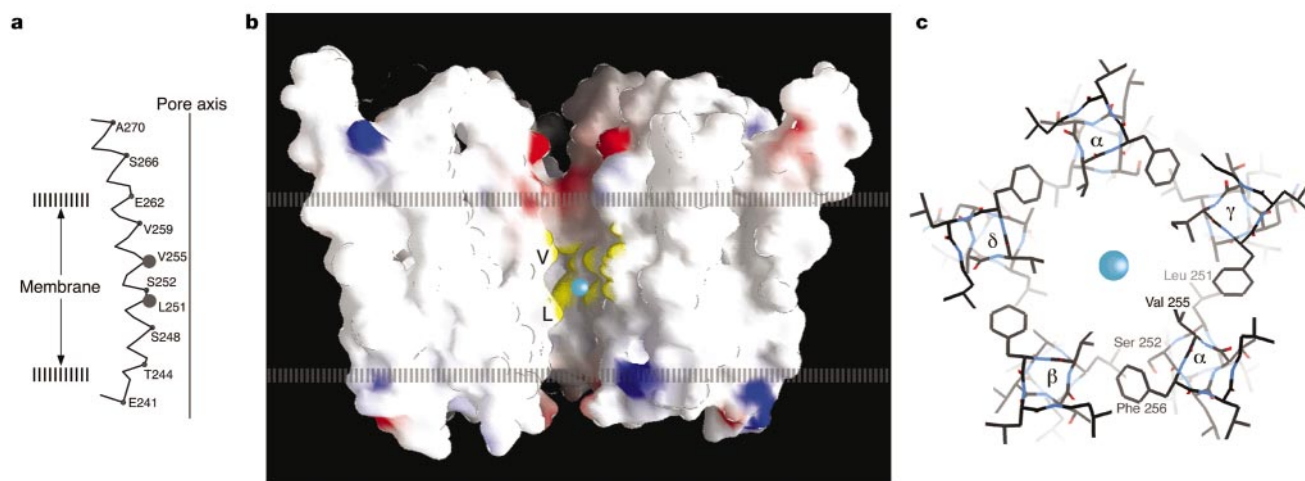


Figure 5 Lining of the pore. **a**, M2 helix showing locations of pore-facing side chains (α -subunit; C α trace). **b**, Molecular surface of the pore domain with front subunit removed, rendered with GRASP⁴⁷. Red and blue correspond respectively to areas of high negative and high positive charge; yellow highlights the hydrophobic area containing the

gate; V and L identify α -Val 255 and α -Leu 251. **c**, Symmetrical arrangement of side chains forming the gate, as interpreted in Fig. 2c. The blue sphere in **b** and **c** is the size of a sodium ion (a hydrated ion may be trapped in this region).

small polar residues (at α -Thr 244, α -Ser 248, α -Ser 252), in the most constricting region of the open pore^{26,27}, are either partly or fully exposed to the ions.

The gate

The pore has special properties in the middle of the membrane. First, it is maximally constricted in this region, owing to the small separation of the M2 helices and to the presence of bulky hydrophobic side chains. Second, it is essentially symmetrical in this region, owing to equal side-to-side hydrophobic interactions between equivalent surfaces of homologous residues (Fig. 5c). The contacts are at two levels: one involving leucine (at α -Leu 251) with the neighbouring alanine (or serine) side chains (at α -Ser 252), and the other phenylalanine (at α -Phe 256) with the neighbouring valine (or isoleucine) side chains (at α -Val 255).

These symmetrical side-to-side interactions bring together the side chains on the neighbouring helices to make a tight hydrophobic girdle around the pore. The minimum radial distance from the central axis to the nearest van der Waals surface²⁸ is close to 3 Å at α -Leu 251 and at α -Val 255, where the pore is narrowest, and less than 3.5 Å over a $\sim$ 8-Å-long hydrophobic zone extending to α -Val 259. This bore is too constricting for a sodium or potassium ion to pass through while retaining its first hydration shell (giving it an effective diameter of $\sim$ 8 Å), and the ion cannot readily lose part of this shell in the absence of polar surfaces that would substitute for water. The girdle therefore creates an energetic barrier to ion permeation across the lipid bilayer²⁹. It is the only such barrier along the conduction path, and there is no protein occlusion (for example, near the intracellular membrane surface³⁰) that would block the flow of ions. Hence this region can be identified unambiguously as the gate of the pore.

The leucine (at α -251) and valine (at α -255) side chains contribute a major portion of the hydrophobic surface of the girdle, and their role as components of the gate is entirely consistent with the interpretation of photolabelling experiments using reagents that penetrate the lumen of the pore^{19,31}. Residues in both rings are labelled efficiently by the small hydrophobic reagent, 3-(trifluoromethyl)-3-(*m*-[¹²⁵I]iodophenyl)diazirine³¹, but only when the pore is closed and the side chains together form a compact hydrophobic binding environment. Moreover, in oocyte expression studies, mutation of the leucine to serine or threonine, in any of the subunits, increases the opening sensitivity of the channel^{32,33}. This

would be expected, as perturbation of the hydrophobic contacts by the polar residues should weaken the girdle and increase the relative stability of the open pore.

Opening mechanism

The three-dimensional fold of the subunits in the ligand-binding domain is built around two sets of β -sheets packed into a curled β -sandwich and joined through a disulphide bridge⁷. By using the AChBP structure⁷ as a template to relate maps derived from electron images, we showed that the two portions of the sandwich, in the α -subunits, convert to an alternative arrangement following activation by a 5-ms exposure to ACh⁸. This allosteric change to open the pore entails rotations of the inner (pore-facing) β -sheets by 15° about an axis passing through the disulphide bridge and oriented normal to the membrane plane⁸.

The spatial relationship of the inner-sheet part of the α -subunit to the membrane-spanning domain was determined by rigid-body fitting of the inner-sheet part to the 4-Å densities (Fig. 2b; see also Methods). Figure 4b shows the two components in the region where they contact each other. The short loop joining the first two β -strands of the inner sheet (β 1/ β 2; α -Glu 43- α -Gln 46; AChBP numbering) is positioned such that the end residue, α -Val 44, docks into the hydrophobic pocket made by the end residues (α -Ser 269- α -Pro 272) of the apposing M2 helix.

Although the limited resolution does not allow a full description, the pin-into-socket interaction between the β 1/ β 2 loop and the end of M2 represents the sole direct link made between the moving elements in the two domains, both of which are found to rotate in the same sense when the receptor is activated^{8,15}. The rotational movements of the inner sheets of the α -subunits are therefore necessarily communicated through this connection and along the pore-lining M2 helices to the gate at the middle of the membrane. For effective coupling, the M2 helices must be free to move relative to the outer protein wall. But this seems a likely possibility because the M2 helices only make limited contacts with the outer wall. Also, both connecting loops begin at conserved glycine residues (Gly 275 and Gly 240; Fig. 4b), lying near the rotation axis, which could confer flexibility by enabling rotational freedom around the peptide bond.

Electron microscopy had shown that the pore widens in the middle of the membrane when the receptor is activated¹⁵—that is, the hydrophobic girdle, forming the gate, comes apart to allow the

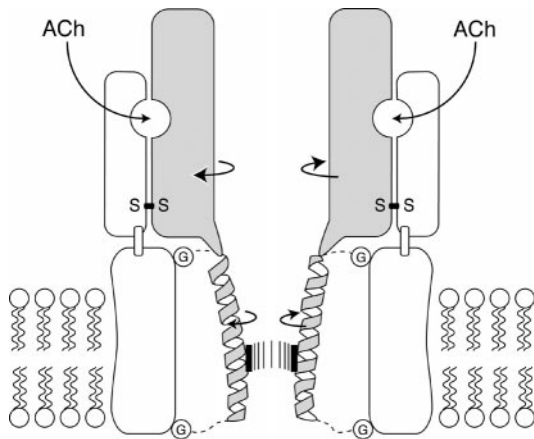


Figure 6 Proposed model for the gating mechanism. The ACh-induced rotations in the α -subunits⁸ are transmitted to the gate—a hydrophobic barrier to ion permeation—through the M2 helices. The rotations destabilize the gate, causing the helices to adopt an alternative configuration which is permeable to the ions. The helices move freely during gating because they are mainly separated from the outer protein wall and connected to it by flexible loops, containing glycine residues (G). S-S is the disulphide-bridge pivot in the ligand-binding domain, which is anchored to the fixed outer shell of the pore. The relevant moving parts are shaded.

ions to flow through. How do the 15° rotational movements of the inner sheets of the α -subunits, communicated to the membrane-spanning domain, destabilize the girdle and open the pore? The structural details imply a simple mechanical model for the mechanism (Fig. 6). First, the allosteric change in the ligand-binding domain, which affects primarily the α -subunits, brings about the rotations of their inner sheets. Second, the rotations of the inner sheets are transmitted by their connected M2 helices to the girdle, or gate, at the middle of the membrane. Third, the twisting movement weakens the hydrophobic side-to-side interactions that hold the girdle together. As a result, the helices ‘collapse’ back against the outer protein wall, making alternative hydrophobic contacts in a configuration that is permeable to the ions.

The integrity of the girdle, like that of other symmetrical assemblies, may depend on equal interactions between each of its components. Thus when only one of these components is sufficiently perturbed, the neighbouring component will lose a set of interactions maintaining its position in the assembly, setting in train cooperative changes that cause the whole structure to flip. The action of just two ligand-binding subunits transmitted to a symmetrical pore may well represent an optimal way to achieve fast and robust gating kinetics, while minimizing the number of activating molecules.

Discussion

The structure of the pore, and our picture of the working protein, are in excellent agreement with the results obtained from several well characterized mutations. Mutations in the pore domain that would affect gating can be divided into three categories: (1) those that influence the coupling between the M2 helices and the ligand-binding domain; (2) those that influence the side-to-side interactions between the M2 helices; and (3) those in other regions that interfere with their movements. An example in category (1) would be the natural mutation of α -Ser269Ile, at the end of M2 next to the inner sheet of the ligand-binding domain (Fig. 4b). This mutation is the cause of a congenital myasthenic syndrome (CMS), and prolongs the apparent open-channel lifetime^{34,35}. However, the lifetime is not altered by the same mutation at equivalent positions in any of the three non- α -subunits³⁵, consistent with the special role played by the α -subunits in transmitting the conformational

change. ϵ -Thr264Pro is another CMS mutant, which gives rise to spontaneous and prolonged channel openings³⁶. It is an example in category (2), because the residue (aligning with α -Thr 254; Fig. 4b) is a component of the girdle, or gate. The introduction of the helix-bending proline at the gate would be expected to weaken it and shift the equilibrium in favour of open events. The CMS mutation α -Val285Ile, which leads to abnormally brief channel openings³⁷, is an example in category (3). α -Val 285 is on the M3 helix close to α -Val 261 on M2 (Fig. 2b), and the larger isoleucine side chain in this position could facilitate interaction between the non-moving (M3) and moving (M2) parts, consistent with the interpretation that the mutant stereochemically inhibits the gating movements³⁷. Finally, several experiments have highlighted the uniqueness of the girdle-stabilizing leucine residue (at α -Leu 251) in relation to the gating mechanism. One observation is that replacement of the leucine by serine increases the opening sensitivity of the channel by the same amount, independent of which subunit is mutated³². This property supports the idea that symmetry is important in determining the integrity of the gate.

Our results from electron images have yielded the first detailed information about the fold and arrangement of the protein chains forming the pore of a transmitter-gated ion channel. They clarify the nature of the gate in the ACh receptor, and suggest how ACh entering the ligand-binding domain triggers pore opening. It is possible that other neuronal ion channels make use of the same physical principles to achieve precise control of ion flux across the lipid bilayer. □

Methods

Specimen preparation and imaging

Tubular crystals were grown from the postsynaptic membranes of *Torpedo marmorata* electric organ in 100 mM sodium cacodylate, 1 mM calcium chloride, pH 6.8, using fish killed in the autumn³⁸. They were applied to pre-irradiated holey carbon grids, having high electrical conductivity, and frozen rapidly by plunging into liquid-nitrogen-cooled ethane. Images were recorded at 4 K in ice over holes, using a 300-kV field emission microscope incorporating a top-entry liquid-helium-cooled stage¹⁰ (magnification, $\times 36,800$; defocus range, 9,000–16,000 Å; dose, ~ 20 electrons Å⁻²).

ACh receptor tubes are helical assemblies of protein and lipid molecules arranged on a p2 surface lattice². We examined four helical families ((-16,6); (-18,6); (-17,5); (-15,7)), with diameters ranging from 770 to 832 Å (Table 1).

Analysis of images

The basic methods for selecting and analysing the images in terms of helical Fourier transforms have been described^{11,38,48}. At higher resolution in the equatorial direction, many of the layer-lines overlap and interfere with one another. But different tubes give rise to different patterns of overlap, because of slight variations in orientation of the surface lattice, and the interference error is largely averaged out after many images have been combined³⁸. Altogether 72 unique overlap patterns, within the four helical families, were identified and analysed. Correction for Ewald sphere curvature was calculated from the helical geometry³⁹.

The tubes contain various distortions that give rise to serious loss of signal at resolutions higher than ~ 10 Å. We corrected for the distortions by dividing the tubes into short segments, fitting successive segments independently to a reference structure, and then adding the segments to the reference data set after the misalignments had been removed¹¹. The reference data set, for each helical family, was thus updated in a cumulative way. In this study the improved reference structure, resulting from addition of many images, allowed the segment length to be shortened to a mean value of 679 Å (compared with 759 Å previously³⁸; see Table 1), without increasing the alignment error, thus improving the accuracy of the correction procedure.

To obtain an appropriate scaling for the amplitudes, we compared the experimental measurements with calculated values, using a model tube having the receptor replaced by just the ligand-binding domain in one case, and by the AChBP structure⁷ in the other. To determine the helical transform of the ligand-binding domain, we calculated the helical density waves, $g(n,r)$, by Fourier–Bessel inversion of the layer-line data^{40,41}, and then back-transformed using only those $g(n,r)$ terms lying within the relevant radial range (indicated by the green box in Fig. 1). The helical transform of AChBP, in the equivalent lattice positions, was calculated from the AChBP coordinates (119B from the Protein Data Bank), using a program written by M. Stowell. Plots of the ratios of the amplitudes in the two Fourier transforms, at equivalent points along the layer-lines, indicated there were two components to the resolution-dependent loss of signal from the receptor: a pronounced fall-off at low resolution, and a more gradual ‘fade-out’ at higher resolution (Supplementary Fig. 2). These effects could be attributed, respectively, to the presence of a proportion of receptors that were not properly ordered on the surface lattice and to imaging deficiencies, such as beam-induced movement and radiation damage⁴². Similar resolution-dependent fall-offs were observed with all helical families. The loss of signal in

each case was compensated by subtracting the low-resolution structure ($<1/15 \text{ \AA}^{-1}$; 84% weight), and sharpening by a temperature factor of $-110 \text{ \AA}^2$ to restore an approximately constant amplitude ratio over the whole resolution range. The rescaling of the amplitude terms was critical in improving the definition of the outermost membrane-spanning α -helices (Supplementary Fig. 3), which were 'swamped out' by the low resolution terms in studies^{9,15} where this correction was not applied.

Structure determination and evaluation

Structures were calculated from each of the four helical families, in the standard way⁴¹, using all significant Fourier terms extending to $4 \text{ \AA}$ (corresponding to the cut-off angle made by the objective aperture in the microscope). To evaluate the reliability and resolution of the three-dimensional maps, we first divided the image data sets of each family into two halves and calculated maps from these halves. Single molecules isolated from the maps were then compared to determine amplitude-weighted phase differences and Fourier shell correlation coefficients in successive annuli of increasing resolution (Table 1; Supplementary Fig. 1). The final density map was a weighted average of the four aligned structures, as before⁸, but with each of the structures being derived from about twice as many images.

The cosine of the phase error in Table 1 gives a measure of the quality of the calculated structures. We find that this parameter, near the resolution limit, is approximately proportional to the square root of the number of receptors averaged, irrespective of the helical family or whether different families have been combined (Supplementary Fig. 4). Our results are therefore limited primarily by the number of receptors averaged, and further studies should benefit from the implementation of automatic methods of data collection and analysis.

Interpretation of the final experimental density map and model building into the densities were performed using the program O⁴². Initially, we applied five-fold averaging to improve the definition of the α -helical backbone structures, and to establish the precise register and orientation of individual (polyalanine) helices. Subsequently, we fitted the M1–M4 helical segments individually to the experimental map, using the protruding regions along the helical densities to identify the largest side chains. This allowed tentative assignments to be made of each amino acid according to the sequence, both along the helices and along the short M2–M3 connecting loops. These assignments were then validated for each subunit by checking their consistency with residues in equivalent locations around the pentamer. PROCHECK⁴⁴ was used to assess and improve the stereochemical correctness of the initial hand-built atomic model, which has not been refined. Rigid body fitting to the densities of the inner sheet of the β -sandwich was done using the real-space refinement in O, and confirmed previous results⁸ (where the estimated alignment error was $\pm 0.9 \text{ \AA}$). Figures were prepared with MOLSCRIPT⁴⁵, SETOR⁴⁶ and GRASP⁴⁷.

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Correspondence and requests for materials should be addressed to N.U. (mas@mrc-lmb.cam.ac.uk). The atomic coordinates have been deposited in the Protein Data Bank with accession code 1OED. The cryo-EM map has been deposited in the 3D EM database with accession code EMD-1044.

Magmatic and amagmatic seafloor generation at the ultraslow-spreading Gakkel ridge, Arctic Ocean

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A high-resolution mapping and sampling study of the Gakkel ridge was accomplished during an international ice-breaker expedition to the high Arctic and North Pole in summer 2001. For this slowest-spreading endmember of the global mid-ocean-ridge system, predictions were that magmatism should progressively diminish as the spreading rate decreases along the ridge, and that hydrothermal activity should be rare. Instead, it was found that magmatic variations are irregular, and that hydrothermal activity is abundant. A 300-kilometre-long central amagmatic zone, where mantle peridotites are emplaced directly in the ridge axis, lies between abundant, continuous volcanism in the west, and large, widely spaced volcanic centres in the east. These observations demonstrate that the extent of mantle melting is not a simple function of spreading rate: mantle temperatures at depth or mantle chemistry (or both) must vary significantly along-axis. Highly punctuated volcanism in the absence of ridge offsets suggests that first-order ridge segmentation is controlled by mantle processes of melting and melt segregation. The strong focusing of magmatic activity coupled with faulting may account for the unexpectedly high levels of hydrothermal activity observed.

Here we describe the initial findings of the international Arctic Mid-Ocean Ridge Expedition (AMORE 2001) that successfully mapped, surveyed and sampled Gakkel ridge in summer 2001, using two research ice-breakers: PFS *Polarstern* from the Alfred Wegener Institute for Polar and Marine Research, Bremerhaven, Germany, and USCGC *Healy*, based in Seattle, USA. The perennial cover of sea ice in the high Arctic had made the Gakkel ridge inaccessible to conventional ships—but this ridge is important to our understanding of the global mid-ocean-ridge system. The Gakkel ridge is of particular interest because of its unique combination of parameters believed to control the magmatic and tectonic behaviour of spreading ridges. It is the slowest-spreading mid-ocean ridge, with full spreading rates as low as 11.0 mm yr^{-1} in the eastern part of our study area¹; it lies within a basin virtually surrounded at close range by continents; its strike is perpendicular to the spreading direction, and hence it contains no major fracture zones along its entire length^{2,3}; its geographical location provides a unique window into the composition of the sub-polar mantle; and its average depth is the greatest of the major ocean ridges.

The ultraslow spreading rate in particular allows tests of theoretical models of the effects of decreasing spreading rate on ocean ridges. Effects (1)–(4) described below would be predicted to become more pronounced with distance along the Gakkel ridge, as the spreading rate decreases by a factor of two over its full length (by 40% within our study area). (1) Previous studies of the very-slow-spreading southwest Indian ridge (SWIR) have led to the suggestion that volcanism decreases dramatically as spreading rate decreases⁴, and that exposures of mantle peridotite would predominate over basalt along the Gakkel ridge. (2) Conductive cooling of

the lithosphere from above might become significant as rates diminish to $<20 \text{ mm yr}^{-1}$ (refs 5, 6), causing cessation of decompression melting at greater depth, lower overall extents of melting, and greater depths of crystallization⁷. (3) At increasingly slower spreading rates and lower extents of melting, basalts should become more heterogeneous in incompatible elements as mantle veins are sampled preferentially^{8–11}. Mantle veins with low melting temperatures such as pyroxenites¹² would become more noticeable in residual peridotites. (4) Linear extrapolation of observations from fast- to slow-spreading ridges suggests that the incidence of hydrothermal activity should decrease, perhaps to zero¹³ at ultraslow ($<20 \text{ mm yr}^{-1}$) rates.

All of these predictions, based on observations from ridges with faster spreading rates, can be uniquely tested using the results reported here, including bathymetric mapping, and rock and water sampling. Geophysical results are reported in a companion paper¹⁴.

Background and previous work

Gakkel ridge stretches 1,800 km across the Eurasian basin of the Arctic Ocean (Fig. 1). To the west the plate boundary passes via the Lena trough and the Molloy fracture zone into the Knipovich ridge. Its eastern end runs into the continental margin of the Laptev Sea, where rifting continues¹⁵ (Fig. 1). Spreading rates decrease from 14.6 mm yr^{-1} (full rate) at the western end to 6.3 mm yr^{-1} in the Laptev Sea¹. Spreading is asymmetric, with slightly faster rates on the south side^{2,16}. Gravity and bathymetry data require the crust to be thin and/or dense¹⁷. Earlier work from submarines during the Science Ice Exercises (SCICEX) programme¹⁸ provided far better bathymetry than had been available previously, and was able to define the overall ridge axis and major bathymetric features from

8° E to 95° E in the eastern part of our study area. The ridge strike is nearly orthogonal to the North America–Eurasia opening direction¹ for most of its length, except for an embayment in the ridge that runs from 32° E to about 80° E (Fig. 1) and appears to be inherited from the conjugate margins of Siberia and Lomonosov ridge¹⁹. This sinuosity results in small deviations (up to about 25°) from orthogonality. The rift is >5,000 m deep in places, with up to 2,000 m of relief on rift valley walls in the west and less relief in the

east, where the rift valley is filled with sediment¹⁷. Despite the slow spreading rate, recent volcanic activity is evident at least at one locality (85° E) from earthquakes^{20,21,22} and images of recent lava flows on the sea floor²¹. The only basalts and peridotites previously recovered and analysed from the Gakkel ridge were small fragments from two box cores^{23–26}.

Results from bathymetry and rock sampling

Three magmato-tectonic domains. Multibeam bathymetry data collected by both ships (Fig. 2), combined with rock recoveries, were necessary to determine the nature of the sea floor and to reveal geologic detail critical to understanding the segmentation and volcanic and tectonic processes of this ultraslow-spreading mid-ocean ridge. Basalt and peridotite were successfully recovered by both ships on AMORE from about 200 stations along 1,000 km of the axis and walls of Gakkel ridge, primarily by dredging. In contrast to pre-cruise predictions, basalt was the predominant rock type recovered over most of the region (Figs 2, 3b). Its limited alteration indicates recent volcanic activity along much of the ridge axis. Basalt and peridotite outcrops could not be predicted reliably on the basis of bathymetric data alone. Magnetic intensity data (J. Brozena, personal communication) and SCICEX sonar backscatter data (M. Edwards, personal communication) correlated more reliably with volcanic areas. The coordinated interpretation of bathymetry and sampling and the earlier data reveals three distinct regimes with different relative abundances of rock types: a western volcanic zone, a central zone that is nearly amagmatic and an eastern zone of widely spaced volcanoes.

Western volcanic zone (WVZ). The WVZ (7° W–3° E) begins at Lena trough, and over a distance of 220 km contains five elongate axial volcanic ridges that are each 15–50 km long and rise 400–1,300 m from the axial valley floor at 4,200 m (Fig. 2). They are separated from each other by short regions containing small volcanic cones. The largest volcanic ridges are located in the centre of the WVZ at 1.5° W and 4.5° W, suggesting some regular long-wavelength variation in magmatic supply punctuated by discrete magmatic centres. The clear volcanic and bathymetric segmentation is remarkably linear, and occurs in the absence of any ridge offsets. Rocks recovered in the WVZ (even from axial valley walls) were virtually all glassy pillow basalts, some of which were very fresh. (Figs 2, 3). This entire segment reflects abundant volcanism. The spreading rate is 14.5–13.5 mm yr⁻¹, but the morphology of its axial valley, such as linear volcanic ridges, is reminiscent of ridges spreading twice as fast (for example, the Mid-Atlantic Ridge (MAR) at 23° N (refs 27, 28), spreading 25 mm yr⁻¹). Rift valley walls consist of a series of inward-stepping normal faults similar to those at other slow-spreading ridges²⁸, and define a rift valley 7–20 km wide. The WVZ terminates eastwards with a scattering of volcanic cones.

Sparsely magmatic zone (SMZ). At 3° E, there is a 10-km, left-stepping, non-transform offset, where the character of the ridge changes (Fig. 2). The axial depth abruptly deepens by 1,100 m, while the bounding rift walls consist of 20° slopes, many of which are defined by large throw fault surfaces >1,000 m. The axial valley walls are 0–4 km apart at their base. There are no volcanic ridges present in the axis, only a gentle 200 m saddle. This axial valley runs 60 km to the northeast, where the axis is offset 15 km to the right (southeast) at 7° E and a similar axial valley continues more than 100 km to the northeast to 17° E.

The abrupt morphological changes that occur east of 3° E are accompanied by large exposures of mantle peridotite (some very fresh), even in the axes of the valleys. No basalts were recovered between 3° E and 8° E. From 8° E to 12° E, peridotites predominated in all axial dredges: rare basaltic rocks were either diabase or old basalt fragments without glass. The abrupt changes at 3° E also coincide with a dramatic decrease in magnetic intensity and free air gravity (J. M. Brozena, personal communication), and confirm

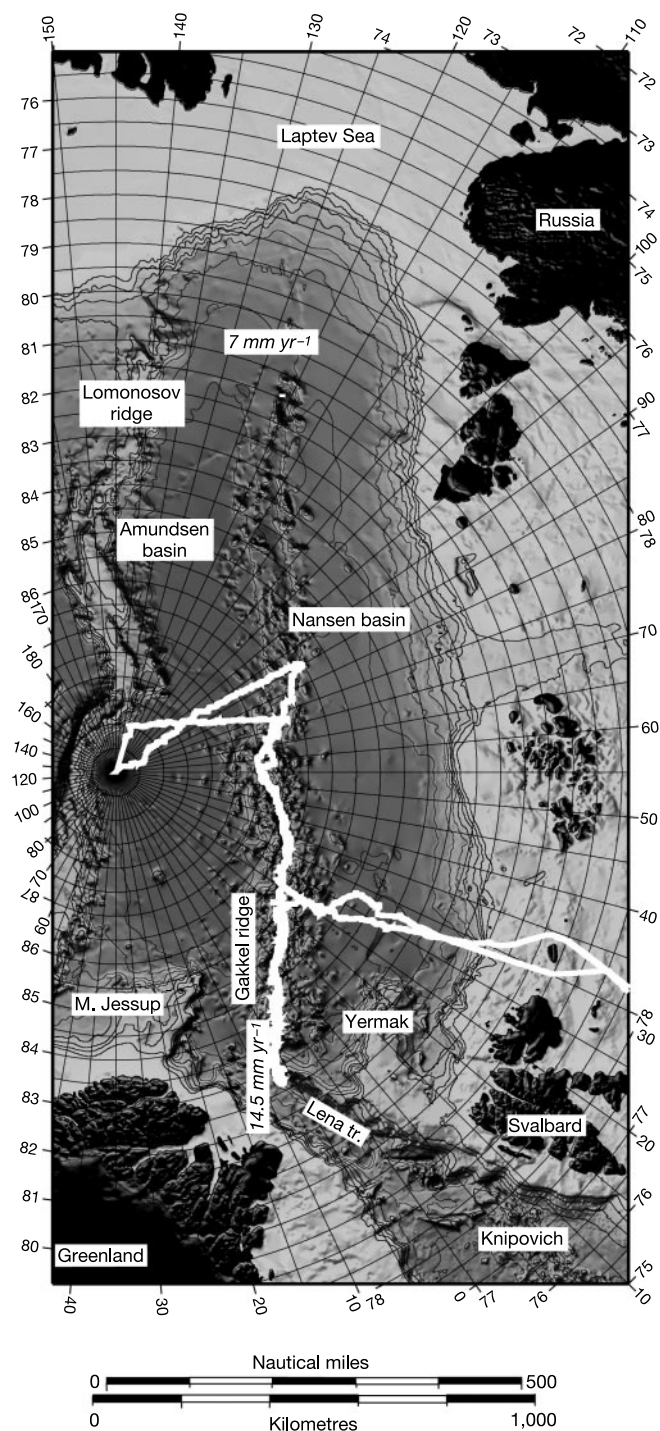


Figure 1 Bathymetric map of the Arctic Ocean. Modified from ref. 49, showing Gakkel ridge and surrounding features. Italicized numbers are full spreading rates, calculated from ref. 1. Black areas are land; light grey areas are continental shelves; dark grey areas are deep ocean basins. The white line shows the cruise tracks of USCGC *Healy* and PFS *Polarstern*.

earlier predictions from magnetic and bathymetric data alone that the thickness of the basalt layer varies along-axis². The dearth of basalts and the absence of any volcanic topographic features suggests that sea-floor spreading here involves little, if any, axial volcanism. Spreading can be amagmatic, with fresh mantle peridotites emplaced directly on the sea floor at the spreading axis.

The first sign of renewed volcanism towards the east is a small volcanic edifice of basalts enriched in incompatible elements at 13° E. A larger volcano then fills most of the rift valley along 40 km of the ridge centred at 19° E (southwest of a much larger seamount), and for another 80 km eastward very little basalt was recovered. For the entire 300-km-long region between 3° E and 29° E, less than 20% of the rift valley appears to be covered by basaltic outcrops, consistent with SCICEX sidescan sonar images existing east of 8° E (ref. 18). Gabbros were also very rare, being minor constituents in only four dredges in this region, consistent with seismic models¹⁴ that suggest that the major plutonic part of normal oceanic crust (layer 3) is poorly developed here.

Eastern volcanic zone (EVZ). The volcanic centres at 13° E and 19° E presage the EVZ (29–85° E), where the ridge is punctuated by six large volcanic centres (31° E, 37° E, 43° E, 55° E, 69° E, 85° E) that extend 15–50 km (typically 30 km) along-strike and are spaced 50–160 km apart (Fig. 2). Edifices are larger and slightly more circular compared to those in the WVZ and to most other mid-ocean-ridge volcanoes. The alignment and fabric of the volcanic centres within the embayment (32°–80° E) are closer to the spreading-normal direction than to the overall strike of the ridge (for example, at 69° E; Fig. 2). Despite their slight obliquity, there are no offsets of the rift valley walls, but there are 8–12-km offsets of the volcanic axis that are completely contained within the continuous axial valley, as at the centres at 37° E and 43° E. From west to east, spreading rate ranges from 12.7 mm yr⁻¹ to 11.0 mm yr⁻¹ in the EVZ.

In contrast with the SMZ to the west, every dredge except one from the EVZ recovered basalt. Five dredges recovered some peridotite along with basalt. Some of the freshest basalts were recovered from the small volcanic centre at 85° E, supporting the proposal of recent activity there^{20,21} (Figs 2, 3). Only altered diabase was recovered from the flanks and upper slopes of the prominent seamounts located at the inside corners of the embayment at 30° E and 61° E (Langseth peak; Fig. 2), showing that the seamounts were probably formed by tectonic uplift rather than by extensive volcanic activity. The low intensity of the axial magnetic high in the tectonized zones between volcanic highs¹⁴ suggests that the basaltic cover between volcanic centres is thin.

Mineralogy of peridotites

Peridotites were recovered primarily from the SMZ between 3° E and 29° E (Fig. 2); they were from the centre and walls of the axial valley, and from off-axis scarps. Some of the axial samples were remarkably fresh, showing very little serpentinization. Most of the hundreds of specimens described while at sea, however, have suffered serpentinization typical of abyssal peridotites. Nonetheless, primary olivine, pyroxenes and spinels are frequently preserved, allowing estimation of primary mineral abundance and textures. Lherzolite and harzburgite occur together in the western part of the SMZ, with lherzolites becoming more abundant to the east. Many of the lherzolites are substantially richer in clinopyroxene and spinel than abyssal peridotites from other ridges^{29,30}. The light colour of spinel in lherzolites suggests that it has low Cr/(Cr + Al). These observations suggest that the extent of mantle melting beneath most of Gakkel ridge is low, in accordance with earlier data from a single peridotite sample²⁶. A few dredges in and near the SMZ contained clinopyroxene-free harzburgites with dark (presumably high Cr/(Cr + Al)) spinels that appear to have undergone much greater extents of partial melting and/or reactive melt transport. Fertile lherzolites were recovered in only two dredges east of 42° E: at 46° 30' E and 64° 48', from the lower part of the southern rift valley walls.

Table 1 Composition of mid-ocean-ridge basalts

Cruise	Hly0102	Hly0102	Hly0102	Hly0102
Sample	D5-2	D62-1	D36-1	D14-10
Longitude	2.16° E	85.03° E	12.33° E	4.44° W
Section	WVZ	EVZ	SMZ	WVZ
SiO ₂	46.42	49.12	50.65	50.52
TiO ₂	2.85	1.11	1.47	1.72
Al ₂ O ₃	15.55	16.97	16.76	15.56
FeO	12.68	7.52	8.45	9.85
MgO	7.38	8.80	7.27	6.52
CaO	10.03	11.51	9.53	10.49
Na ₂ O	2.82	3.16	3.92	3.36
K ₂ O	-n.d.-	-n.d.-	-n.d.-	-n.d.-
Total	97.73	98.19	98.05	98.02
Sr (p.p.m.)	274	208	213	174
Ba (p.p.m.)	18.2	17.9	257	69.4
Fe _{8.0}	11.66	8.22	7.32	7.61
Na _{8.0}	2.61	3.37	3.66	2.84

All values except Ba and Sr are in wt%. Analyses by direct current plasma emission spectrometry³¹ (K. Lehnert, Analyst). K₂O not determined.

Chemical compositions of basalts

More than 100 chemical analyses of glasses performed at sea by techniques established previously³¹ allow a first-order description of the major and trace-element systematics of Gakkel ridge basalts. The major and trace elements vary systematically with respect to the magmato-tectonic segments described above.

In the WVZ, the Ba/TiO₂ ratio (an indicator primarily of the enrichment of the mantle source in incompatible elements) is highest at the western end (7° W) and declines steadily eastward toward very low values adjacent to the SMZ (3° E) (Fig. 3). In the SMZ, Ba/TiO₂ becomes highly variable, extending to much higher values; similar to those of the most enriched mid-ocean ridge basalts (E-MORB). Ba/TiO₂ gradually declines eastwards within the SMZ to a narrower range of fairly constant values in the EVZ. The mean Ba/TiO₂ for the entire study area is significantly higher than average MORB (Gakkel mean Ba/TiO₂ = 34, East Pacific Rise (EPR) mean Ba/TiO₂ = 10; 85% of Gakkel samples have more than 20 p.p.m. Ba, in contrast to 33% of EPR samples). The average Gakkel basalt is therefore mildly enriched.

On the basis of their Mg numbers, glasses from the WVZ have undergone greater amounts of crystal fractionation on average compared to those from the EVZ and SMZ (Fig. 3). (Mg number = 100*Mg/(Mg + Fe²⁺), with all values in moles. Mg number is higher for hotter, more primitive, less-fractionated liquids). The average Mg number for the WVZ is 61.6 ± 3.0 compared to 65.7 ± 4.3 for the EVZ, which is similar to the most primitive portions of the MAR (33–40° N; Mg number = 63.9 ± 2.8)³².

Basalts with unique compositions have erupted on the western side of the SMZ. Primitive glasses with relatively high MgO contain high FeO, TiO₂ and Sr, and low SiO₂ and Ba. Their low SiO₂ and exceptionally high FeO (Table 1) suggest that the melts segregated at high pressure compared to most MORB¹¹. Their high TiO₂ and Sr,

Figure 2 Bathymetric map of Gakkel ridge showing lithology of recovered rocks. ► See text for details. Bathymetric maps were produced using the hull-mounted multibeam sonars (Seabeam 2112 on *Healy* and the Hydrosweep DS 2 on *Polarstern*), and were navigated using GPS; data could be acquired even when the ships were breaking ice. Raw data from both ships were combined, and were gridded with a spacing of 150 m. The total surveyed region (inset) covers ~1,000 km along axis from 8° W (Lena trough) to 87° E, providing the first data for the western 300 km of Gakkel ridge. The resolution and navigation of these data are significantly better than charts made previously for part of the area (SCICEX)¹⁸. Coloured symbols show lithology recovered in each dredge. Red, basalt; green, peridotite; orange, gabbro; blue, diabase. The three different zones of the ridge described in the text are separated by the heavy dashed lines. The western volcanic zone (WVZ) terminates at the eastern end of panel **a**. The sparsely magmatic zone (SMZ) then continues to the eastern end of panel **b**. The eastern volcanic zone (EMZ) includes the eastern end of panel **b** and well as panels **c** and **d**.

accompanied by relatively low Ba, suggests they formed by very low extents of melting of a source that was highly depleted in incompatible elements. To the east of the SMZ, compositions in the EVZ become much more regular, particularly in on-axis lavas from the volcanic centres.

The Gakkel ridge holds particular interest because its great depths

and slow spreading rates extend the range of spreading parameters that can be compared to the global database of MORB chemistry for ocean ridges. The Gakkel ridge is consistent with global systematics in terms of $\text{Na}_{8,0}$ (Na_2O normalized to 8% MgO , suggested to be an index of partial melting and which correlates positively with axial depth for the global system of ocean ridges³³). The sectional averages for the Gakkel ridge all fall at the high- $\text{Na}_{8,0}$ end of the global correlations of $\text{Na}_{8,0}$ versus depth and $\text{Na}_{8,0}$ versus $\text{Fe}_{8,0}$ (ref. 33). The shallower WVZ has the lowest $\text{Na}_{8,0}$, and the deeper SMZ the highest. The EVZ has higher $\text{Na}_{8,0}$ and lower $\text{Fe}_{8,0}$ than the WVZ.

Discussion

These observations from the Gakkel ridge provide important new constraints on many aspects of mid-ocean-ridge volcanism.

The effect of spreading rate on ocean-ridge processes. On the basis of observations and extrapolations from faster-spreading ridges^{4,13}, of theoretical modelling of the melting process^{5,6}, and of crustal thermal structure³⁴, Gakkel ridge would be predicted to have sparse volcanism, a preponderance of peridotites over basalts, very little hydrothermal activity, and low extents of melting of the underlying mantle. All of these effects should become more pronounced towards the east as the spreading rate decreases by nearly 40%. Many of the data from the AMORE cruise confound these predictions, demonstrating the important role of other variables in addition to spreading rate in controlling ridge processes.

Relative to the SMZ and EVZ, the WVZ is in a robust magmatic stage. No peridotite was recovered, despite spreading rates lower than the SWIR where peridotites were recovered in nearly one-third of all dredges³⁵. The WVZ is shallower than other portions of the Gakkel ridge, and the lower values of $\text{Na}_{8,0}$ suggest a larger extent of melting compared to SMZ and EVZ. One possibility is that the magmatic vigour of the WVZ compared to the SWIR and the EVZ reflects the influence of a hotspot. Earlier studies suggested that the Morris Jesup rise to the west and Yermak plateau to the east (both of which are about 200 km distant), were created by a hotspot at 35 Myr ago³⁶, but a more recent study³⁷ indicates that no hotspot was present. The robust magmatism of the WVZ is much greater than would have been predicted from its spreading rate, which is a factor of two less than the MARK area at 22° N on the MAR. The SMZ to the east has little magmatism. The transition from WVZ to SMZ might be thought to reflect the expected signal of progressively decreasing magmatism and extents of melting eastwards as spread-

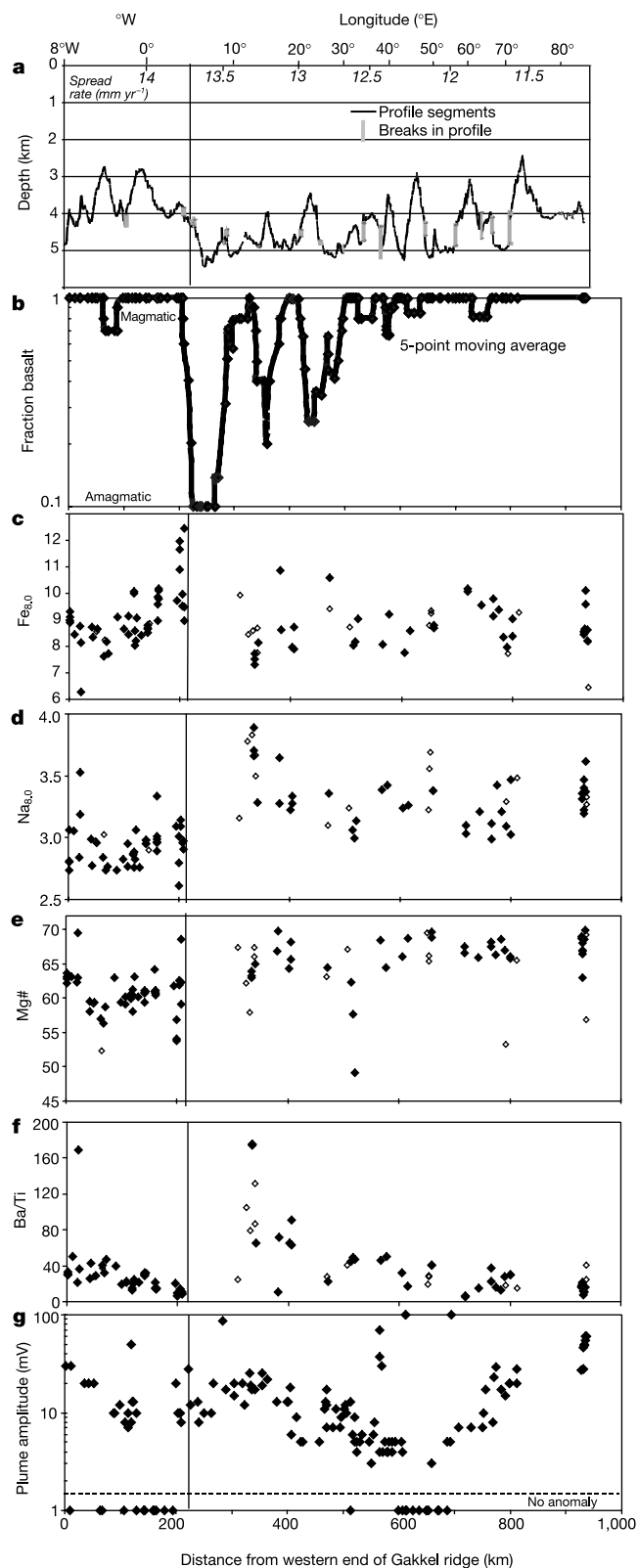


Figure 3 Variation in axial depth, lithology and basalt geochemistry along the axis of Gakkel ridge. Spreading rates shown in **a** were calculated using parameters from ref. 1. **a**, Bathymetric curve, constructed by picking depths every 2 km at the low points between rift valley walls. **b**, Lithological variation, calculated by using the average makeup in each dredge by weight and then taking a running boxcar average for five dredges along axis. **c**, **d**, $\text{Na}_{8,0}$ and $\text{Fe}_{8,0}$ are the weight per cent Na_2O and FeO corrected for fractionation to 8% MgO (ref. 33). $\text{Na}_{8,0}$ correlates positively with axial depth for the global system of ocean ridges and is an index of the extent of partial melting³³, while $\text{Fe}_{8,0}$ indicates the depth of partial melting. **e**, $\text{Mg\#} = 100[\text{Mg}/(\text{Mg} + \text{Fe}^{2+})]$, where all values are in mole proportions and $\text{Fe}^{2+} = 0.9\text{Fe}_{\text{total}}$. It is a measure of the degree of fractionation, and a crude indicator of the eruption temperature of the glasses (lower values are more fractionated with cooler temperatures). **f**, $\text{Ba/Ti} = (\text{p.p.m. Ba})/(\text{wt\% TiO}_2)$, and indicates the enrichment of the mantle source in incompatible elements. Natural basalt glasses were analysed on board *Healy* by direct current plasma emission spectrometry³¹. Filled symbols are samples recovered on-axis, defined as within 5 km of the axial rift, while open symbols were recovered from 7–15 km off axis. In some places the choice of the axial location was subjective. Vertical line indicates the boundary between the magmatic western zone and the SMZ. **g**, Hydrothermal plume amplitude, defined as the maximum signal in millivolts above background observed with the MAPR (Miniature Autonomous Plume Recorder) light-scattering sensor. All values above the dashed line indicate the presence of a significant above-bottom plume. Points below dashed line have no anomaly (zero value), but are plotted at 1.0 to permit the use of a logarithmic scale. Values >100 (up to 110, maximum) are plotted at 100.

ing rate declines. But such a trend does not continue to the east, since the SMZ is sandwiched between zones of higher volcanic output on both sides. Hence factors in addition to spreading rate clearly play a role in determining magmatic vigour. Ridge obliquity has also been suggested to reduce magmatic output via decreased mantle upwelling, as along the SWIR, where there is a highly oblique amagmatic supersegment³⁸. But along the Gakkel ridge, the oblique zone (31° E–80° E in the EVZ) is more volcanically active than the axis to the west (SMZ: 3°–29° E), despite the fact that there is both decrease in spreading rate and increase in obliquity. The fact that the SMZ has exactly the same strike as the WVZ also shows that obliquity is not an important controlling factor in this region.

The dying out of melting in the SMZ is apparent from both the lack of magmatic activity (Fig. 3b) and the chemistry of the basalts at the western margin of the SMZ which suggests very small extents of melting of a depleted source (Table 1 and Fig. 3c–f). The lack of magmatism must reflect either lower mantle temperatures and/or a more depleted or refractory mantle composition in this region. The depleted harzburgites in the SMZ may represent the residues of extensive melting in a prior melting event that made them infertile. In this case, their compositions might not reflect the current melting conditions beneath the axis³⁹. Alternatively, some or all of them could represent melt flow channels⁴. Although more detailed geochemical work on basalts and peridotites is necessary, there is evidence that both a lower mantle temperature and a less fertile source are responsible for the paucity of magmatism in the SMZ.

The basalts provide some evidence to confirm predictions that ultraslow spreading should lead to colder temperatures with depth, and therefore to thicker lithosphere⁵ because less heat is advected to the surface. The low SiO₂ at high FeO for basalt glasses from the western margin of the SMZ, and the deep segregation depths inferred for them, are consistent with thicker lithosphere from this region as compared to faster-spreading ridges¹¹. We propose that the large variability in magma production and melting along Gakkel ridge may be enhanced by a thick overlying lithosphere. With thicker lithosphere, the vertical dimension of the melting regime is diminished. Small along-axis variations in mantle temperature or mantle composition then lead to greater proportional changes in the vertical dimension of melting and therefore the extent of melting and magma production, because the length of the melting column on which these changes operate is far less.

Implications for the veined-mantle hypothesis. Gakkel ridge basalts and peridotites provide new perspectives on whether the sub-oceanic mantle is ubiquitously veined. If veins are present, the mean enrichment of basalts should increase progressively as the extent of melting decreases^{8,40}. This may account in part for the overall enrichment of mean compositions along this ridge. However, basalts from the EVZ are not as enriched as those from the WVZ, even though they appeared to have formed by smaller extents of melting. More important evidence comes from the samples from the western boundary of the SMZ (Table 1: D5–2). These melts formed by extremely low degrees of melting, yet they are not enriched, as would be predicted from the veined-mantle model. On the other hand, the presence of some veins in discrete regions is supported here, as elsewhere, by the high Ba/TiO₂ basalts present in the west and within the SMZ. However, the geochemistry of these samples suggests metasomatic enrichment, and not recycled eclogite.

Gakkel peridotites also lack direct evidence for greater preservation of primary mantle veins. The abundance and nature of sparse pyroxenite veins is similar to residual abyssal peridotites from faster-spreading ridges. Veins of gabbro and of plagioclase-bearing peridotite are not primary veins, but instead are evidence of low-pressure melt impregnation, and suggest that low degrees of mantle melting have occurred. Together these observations suggest that primary mantle veins were rarely present, or that they melted out at very low degrees of partial melting.

Ridge segmentation and overall architecture. The Gakkel ridge permits a clear separation of variables, because magmatic segmentation at a large scale similar to that of transform-bounded segments occurs in the absence of any tectonic segmentation or offsets, especially on the WVZ. These data show unambiguously that ridge segmentation can be controlled by processes of magmatic segregation in the upper mantle, and does not require tectonic segmentation imposed from the lithosphere⁴¹. Processes of melt segregation in the mantle can lead to discrete loci of magma delivery⁴², and melt focusing can occur in the absence of tectonic influences (for example, fracture zones or offsets), as it does in island arcs, continental rifts and nascent spreading centres⁴³. The ultraslow spreading rate may foster focusing of partial melts from mantle upwelling⁴⁴.

Abundance of hydrothermal activity. Another contrast with previous conceptions of the role of spreading rate on ridge processes is reflected in the frequency with which we observed hydrothermal plumes in the water column (Figs 3g, 4)⁴⁵. The fraction of the axis that is affected by plumes may be enhanced (relative to the number of discrete vent sites) by hydrographic features and the water column density structure in this region. First, the very deep rift valley may enclose the plumes in closed basins, which would permit them to persist longer than in an unconfined ridge axis. Second, the unsegmented nature of the rift valley may allow plumes to disperse further along axis than typical. Third, the very cold temperatures and relative vertical uniformity of Arctic deep water may render the hydrothermal plumes both easier to discern and more persistent. These possibilities show that it is not straightforward to calculate hydrothermal vent frequency from plume incidence.

Although the observed plume incidence of ~80% may be thus inflated by unique local conditions, the inferred occurrence of hydrothermal sites along Gakkel ridge (12 sites in 1,100 km) is also greater than predicted for this spreading rate, by a factor of at least two to three⁴⁵. Unlike the abundant ultramafic-hosted hydrothermal deposits recovered from the ultraslow SWIR⁴⁶, the venting on Gakkel ridge appears to be localized at volcanic centres: for instance, every volcanic centre in the EVZ hosts a vent site. Although plume incidence is high in the SMZ, only one site can be located in this region with confidence and the extent of peridotite-hosted venting remains unknown. The trade-off between magmatism and faulting in determining vent location has been emphasized on a

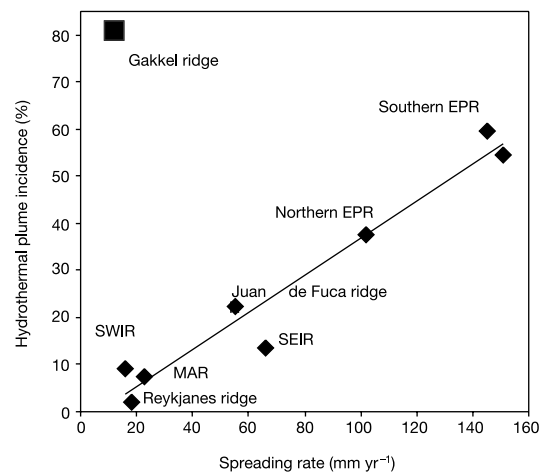


Figure 4 Correlation of spreading rate and incidence of hydrothermal plumes. Adapted from ref. 13 and including the Gakkel ridge estimate, which was calculated as the percentage of deployments of the MAPR for which an anomaly was recorded⁴⁵. The plume incidence for Gakkel ridge is much higher than predicted by extrapolation from faster ridges, as a result of both vent frequency and hydrographic conditions (see text). SEIR., southeast Indian ridge

local basis in several places around the ocean-ridge system^{47,48}. On Gakkel ridge, focusing of mantle melts leads to large volcanic centres where the heat necessary to fuel hydrothermal systems is concentrated. The ultraslow spreading rate fosters deep, long-lived faulting that permits access to the heat—especially important if magmatism is episodic in an area—allowing new volcanic heat sources develop within older, fractured lithosphere. The combination of these geologic and hydrographic characteristics leads to a more complex relationship than predicted between plume or vent incidence and spreading rate at this end of the global ridge spectrum¹³.

In general, these results show that spreading rate and tectonic segmentation alone are not the defining variables for the operation of ocean ridge systems. At the slowest spreading rates for the ocean ridge system, magmatism was far more robust than predicted and peridotites were rare along most of the ridge. Bathymetry, tectonics and magma chemistry did not vary progressively along axis as the spreading rate decreased, but showed more complex relationships, requiring control by other variables. Hydrothermal activity was far more abundant than predicted on the basis of previous correlations with spreading rate, highlighting the complexity of the interplay between magmatic, tectonic and hydrographic variables in determining plume incidence. Segmentation appears in the volcanic system independent of tectonic offsets, showing unequivocally that segmentation can arise from melting and melt segregation processes in the mantle alone. The multi-variable aspect of the ocean ridge problem is also indicated by the contrasting evidence for the veined-mantle hypothesis. There is evidence for no veining in some regions, and possible veining in others.

Investigation of the ultraslow endmember of the ocean ridge system therefore clearly documents the importance of variables other than spreading rate for all aspects of the behaviour of ocean ridges. A multi-dimensional perspective will be essential for quantitative modelling of the diversity of ocean ridge processes. □

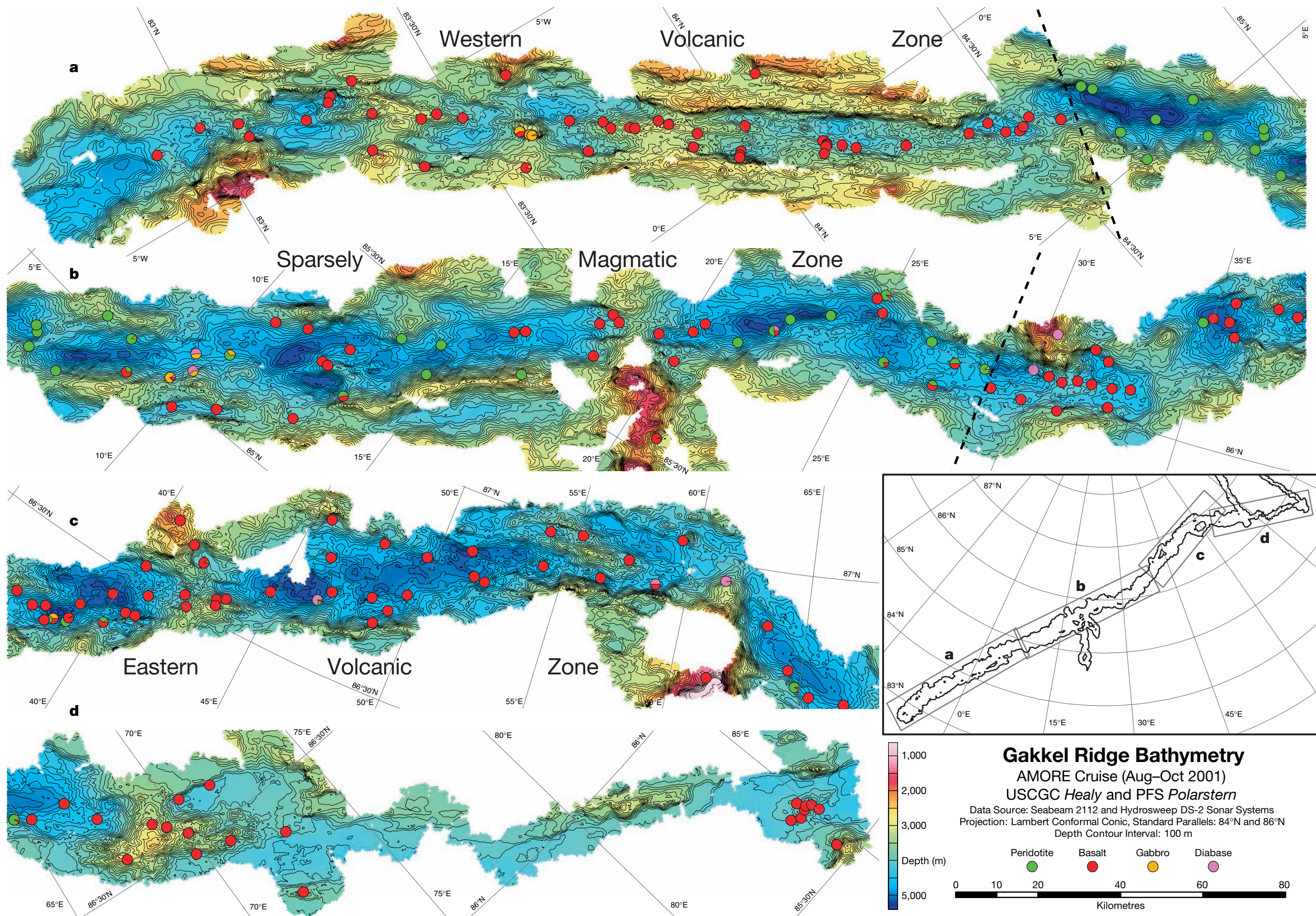
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Geophysical evidence for reduced melt production on the Arctic ultraslow Gakkel mid-ocean ridge

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Most models of melt generation beneath mid-ocean ridges^{1–3} predict significant reduction of melt production at ultraslow spreading rates (full spreading rates $<20 \text{ mm yr}^{-1}$) and consequently they predict thinned oceanic crust. The 1,800-km-long Arctic Gakkel mid-ocean ridge is an ideal location to test such models, as it is by far the slowest portion of the global mid-ocean-ridge spreading system, with a full spreading rate ranging from 6 to 13 mm yr^{-1} (refs 4, 5). Furthermore, in contrast to some other ridge systems, the spreading direction on the Gakkel ridge is not oblique and the rift valley is not offset by major transform faults. Here we present seismic evidence for the presence of exceptionally thin crust along the Gakkel ridge rift valley with crustal thicknesses varying between 1.9 and 3.3 km (compared to the more usual value of 7 km found on medium- to fast-spreading mid-ocean ridges). Almost 8,300 km of closely spaced aeromagnetic profiles across the rift valley show the presence of discrete volcanic centres along the ridge, which we interpret as evidence for strongly focused, three-dimensional magma supply. The traces of these eruptive centres can be followed to crustal ages of $\sim 25 \text{ Myr}$ off-axis, implying that these magma production and transport systems have been stable over this timescale.

The oceanic crustal thickness measured seismically can generally be regarded as a first-order measure of the total mantle melt production. For this reason, the Gakkel ridge is the key region for testing at ultraslow spreading rates the relationships of spreading rate versus oceanic crustal thickness predicted by melt generation models^{1–3}. A small number of seismic investigations from ice stations located off-axis on older oceanic crust from Gakkel ridge indicate crustal thicknesses between 2 and 6 km (refs 6–9), but it is not known if these are typical of the current rift valley. Recent gravity models across the Gakkel rift valley suggest thin crust, with a minimum of 3–4 km for the western part between 7° W and 70° E (refs 10, 11). However, this part of the Gakkel ridge remained poorly investigated until the joint AMORE expedition of the research ice-breakers PFS *Polarstern* and USCGC *Healy* during August and September 2001 (see also ref. 12). Seismic refraction, heat flow and bathymetric investigations were performed along this sector¹³ (Fig. 1). Seismic refraction experiments were carried out at 18 locations using a 24-litre airgun array towed behind PFS *Polarstern* and receivers deployed on ice floes. To supplement the seismic data, detailed aeromagnetic investigations were conducted with helicopters mainly in two areas (Figs 1, 2, 3).

Four seismic refraction stations were deployed to give direct constraints on the crustal thicknesses of the two areas investigated (Fig. 1). Owing to the ice conditions, the profiles are not straight lines, but follow in general the strike of the rift valley. For the two-dimensional ray-tracing¹⁴ models, the bathymetry acquired during the survey was incorporated (see Supplementary Information).

Within both magnetic survey areas, the seismic stations (Figs 2, 3) indicate very thin oceanic crust between the off-axis ridges B and

D. Here, seismic velocities with a linear velocity gradient from 3.0 to 4.8 km s^{-1} are observed for the upper portion. The velocities of this unit are constrained at several stations of the survey, and are confirmed as typical by other experiments^{15,16}. This section is interpreted as basaltic lavas and dykes. Locally, at a depth of approximately 1–2 km below the sea floor, seismic velocities increase to 5.0 – 6.4 km s^{-1} at some stations (for example, station 232; ridge D). Crustal velocities typical of oceanic layer 3 (6.5 – 7.2 km s^{-1}) are missing in the data. Thus, this oceanic layer is either not present at all, or cannot be resolved by the experimental set-up.

Clear P_n phases (representing waves refracted from the top of the mantle) with velocities between 7.5 and 7.8 km s^{-1} are observed at well over 5–15 km distance in most record sections, and provide excellent constraints for the crustal thickness. These velocities most probably represent partly serpentinized upper-mantle peridotites. The record sections (Figs 2, 3) show crustal thicknesses ranging between 1.9 and 3.3 km. In general, the crust appears to be constantly thin in the segments investigated. There is no clear indication in the seismic data that the crust is locally thickened where ridges B and D intersect the axis. Seismic velocities between 4.8 and 6.0 km s^{-1} , however, are present below ridge D, which might indicate the presence of a larger amount of basaltic material due to an increased magma supply. These basalts are less affected by fracturing and alteration processes over the depth range studied and, thus, have higher seismic velocities. The recording stations in the basins (Figs 2, 3) show extremely slow seismic velocities of less than 4.8 km s^{-1} for the entire crustal section, in a region where mainly variably serpentinized peridotites were dredged¹².

Changes in the crustal composition can best be understood by including the AMORE¹² and IBCAO¹⁷ bathymetry in the interpretation (Fig. 1). The general features of the investigated part of Gakkel ridge are briefly described here (details in ref. 12). West of $3^\circ 30' \text{ E}$, the bathymetry is dominated by elongated volcanic features composed of numerous tiny volcanic cones with stepped rift valley walls and short-throw faults. East of $3^\circ 30' \text{ E}$, the shape of the median valley changes radically. It becomes more rugged, and deepens significantly from 4,000 m in the west to 5,200 m. Starting at 10° E , the Gakkel ridge is cut by several basement ridges perpendicular to its strike with a spacing of $\sim 100 \text{ km}$. Six ridges (A–F) occur between 10° E and 70° E along the rift valley. Slight changes in the direction and depth of the rift valley are associated with four ridges at 19° E (B), 31° E (C), 62° E (E) and 70° E (F). At 10° E (A), the basement ridge is not well developed. At ridge D (42° E), no major change in strike of the rift valley occurs.

Detailed aeromagnetic surveys were conducted to understand better the nature of the basement ridges. These data help to constrain the crustal structure, because the amplitude of the magnetic anomalies is strongly influenced by the strength and distribution of the magnetized rocks in the upper lithosphere, and thus may indicate changes in crustal construction that are not visible in the velocity models. In the first study area (Fig. 2), strong magnetic amplitude variations are visible from southwest to northeast within the axial valley. At the intersection of ridge B with the rift valley, the central magnetic anomaly peak has a maximum of 520 nT and almost vanishes along axis towards the northeast. Across the southern flank of basement ridge C, the magnetic anomaly increases again to 400 nT. The northern flank of ridge B is characterized by negative spreading anomalies of up to 300 nT followed by smooth positive ones. The southern flank of ridge B shows no such continuity of the magnetic anomalies. Off-axis between ridges B and C, the magnetic signature on both flanks is indistinct, becoming more pronounced again as ridge C is approached.

The second area (Fig. 3) is located 100 km further to the northeast at 41 – 50° E . Here, the magnetic data strip is 54 km long and partly

covers basement ridge D. The bathymetrically subdued rift valley is characterized by a strong positive magnetic anomaly up to 580 nT. Towards the northeast, the anomaly decreases to 220 nT and becomes broader. At 47°30' E, the magnetic data indicate a 13-km offset of the neo-volcanic zone, which is not bathymetrically expressed. North of this offset, the axial anomaly increases from 0 to 200 nT. Though the water depths in the median valley are up to 2,000 m greater than along the flank, the central magnetic anomaly is still the strongest.

The variations in the magnetic field are strongly correlated with the existence of the basement ridges A–E (Fig. 1). The strongest

magnetic anomalies of our survey are found over the ridges and indicate more highly magnetized crust, consistent with a larger amount of magnetized material in the crust or trapped melts in the mantle.

In the previous magnetic data set acquired in the 1970s, these basement ridges were not resolved in such detail^{4,5}. Off-axis, a continuous magnetic anomaly pattern was found, not displaced by any large-scale fracture zones at the rift valley. On the basis of the published magnetic age model⁵ and the IBCAO bathymetry¹⁷, ridge E, for example, can be followed 110 km off-axis to magnetic chron 6 (20–25 Myr ago). The rift valley expressions of these ridges, and the

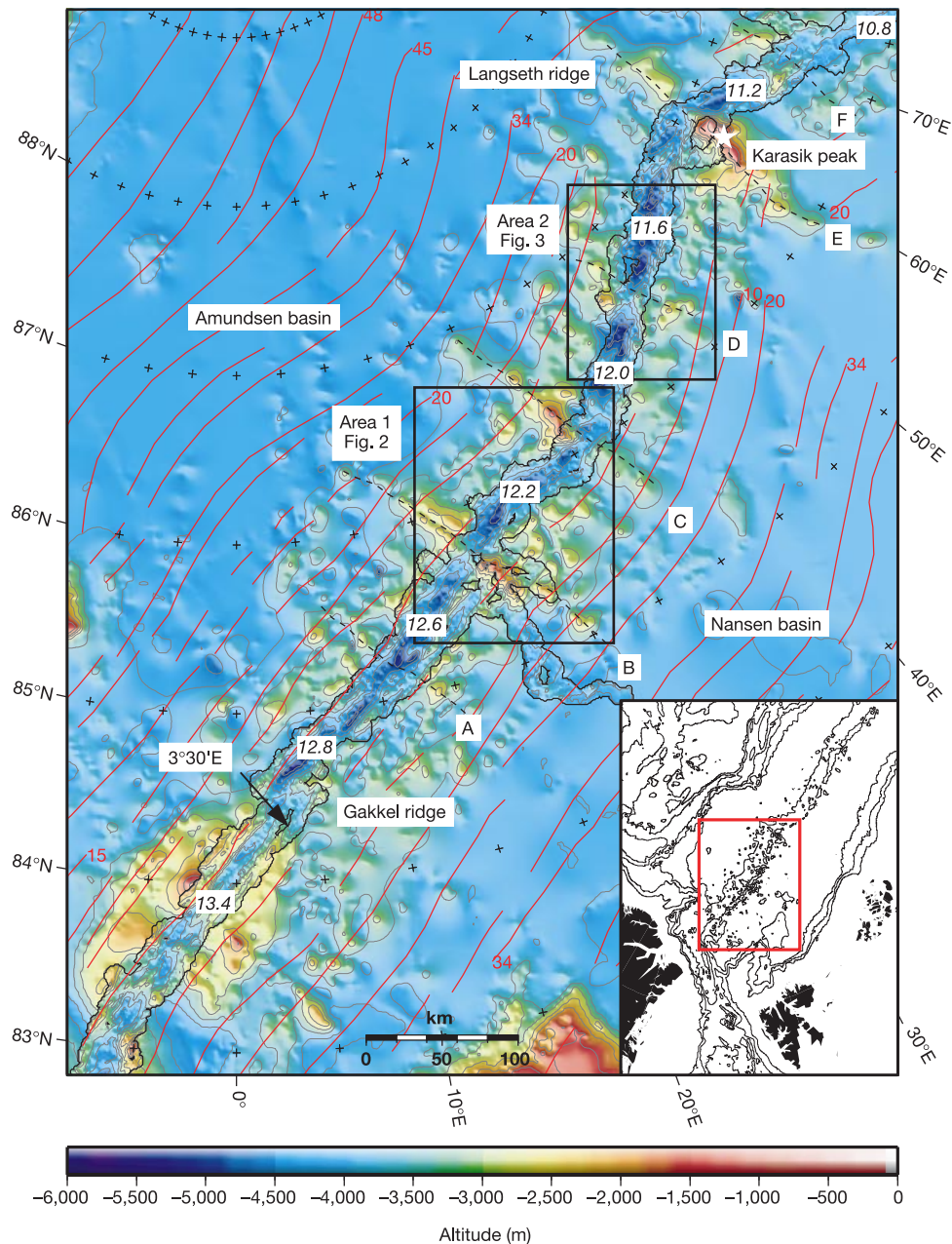


Figure 1 Map of combined IBCAO¹⁷ and AMORE¹² bathymetry for the Arctic Ocean. The study area is shown in the inset. The area of the high-resolution multi-beam bathymetry acquired by PFS *Polarstern* and USCGC *Healy* is indicated by a bold black line. Pronounced basement ridges perpendicular to the rift valley are indicated by dashed lines and labelled A–F. The full spreading rates (mm yr^{-1} ; shown in italics) based on the

NUVEL-1¹⁸ model are marked along the rift valley. The areas of detailed magnetic surveys are marked with black boxes. The red lines indicate magnetic isochrons⁵, and the numbers on the isochrons show the ages in Myr. In Figs 1–3, ‘altitude’ on the colour bar shows the position of the sea floor with respect to sea level.

off-axis portions that were mapped, clearly show their volcanic nature. Dredging on- and off-axis on these volcanic ridges returned exclusively volcanic rocks¹². However, accreted crust is still present in the deep basins between the basement ridges along the axial valley indicated by small volcanic centres visible in the high-resolution Parasound 4-kHz profiler records¹³.

We interpret the basement ridges to be a result of focused and increased melt supply in an ultraslow spreading regime. The existence of spreading anomalies along these basement ridges seen in the new magnetic data (for example, ridge B, Fig. 2) implies that this style of accretion persisted for several million years. The decrease in amplitude of the central rift valley magnetic anomaly might indicate a lateral melt migration toward the volcanic centres. The melt supply rate beneath the eruption centres east of 3° 30' E is not sufficient to permit along-axis migration of magma

along the investigated part of the ridge. Dredging of the basins returned at a few locations only variably serpentinized peridotites¹² (Figs 2, 3).

Our seismically determined crustal thicknesses along the rift valley of Gakkel ridge are well below what has been found for the Mohns ridge¹⁵ (4 km at 14 mm yr⁻¹), but are comparable with experiments along the southwest Indian ridge (SWIR)¹⁶ (≥ 2.5 km at 12 mm yr⁻¹). The velocity–depth functions along the Gakkel ridge are similar to both regions, having low seismic velocities close to the sea floor and a reduced or missing layer 3. A pronounced increase of the seismically determined crustal thickness below the basement ridges B–D is not observed. Thus, the region of melt supply beneath the basement ridges in the rift valley in the area east of 3° 30' E must be either very narrow, or its effects too small to be visible in the seismic data. These observations strongly support theoretical models that predict thin oceanic crust for spreading rates < 20 mm yr⁻¹ (refs. 1–3). East of 3° 30' E, non systematic crustal thickness variations of almost 50% along the rift axis with only slightly decreasing spreading rate (10%) indicate that the crustal thickness is not only a function of spreading rate, but also depends on the strong three-dimensional character of ridge magmatism.

The most surprising result of this study is the change in tectono-magmatic style of Gakkel ridge with spreading rate along strike. The long western segment has a subdued magmatic segmentation and topographic characteristics similar to much faster-spreading ridges¹². It has a robust central magnetic anomaly of up to 700 nT at 3° E, where the spreading rate of the ridge is fastest (13 mm yr⁻¹).

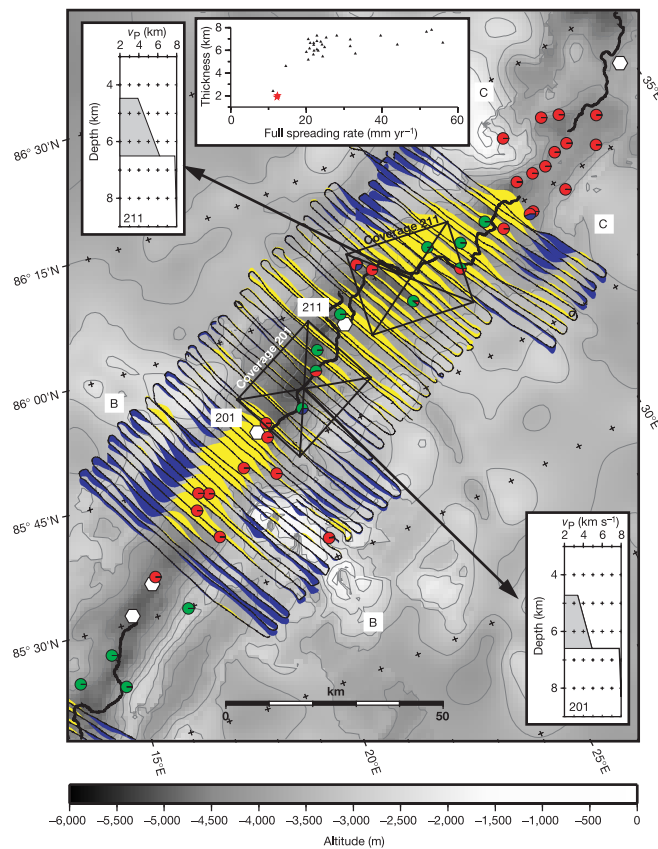


Figure 2 Map of survey area 1. The black line in the median valley shows the ship tracks of both research ice-breakers during seismic profiling. The station labels (white hexagons, some with numbers) indicate the positions of the seismic recording stations at the start of the survey. For two stations a one-dimensional velocity–depth function (light grey, velocities < 4.8 km s⁻¹; dark grey, velocities > 4.8 km s⁻¹, station number indicated in lower left corner) is displayed. v_p , P-wave velocity. The rectangles indicate the area for which the calculated crustal thickness is valid. Aeromagnetic lines are shown perpendicular to the rift valley (yellow, positive anomaly; blue, negative). These have a spacing of ~ 2 km and are 50–75 km long. The magnetic sensor was towed 35 m below the helicopter at a flight level of about 100 m. The data have been processed in a standard fashion; that is, edited, IGRF corrected and upward-continued to an altitude of 500 m. Magnetic data from NyÅlesund, Svalbard are used for the corrections of the diurnal variations. The bathymetry is taken from the AMORE/IBCAO data set. Gridding cells are 1 km and contour intervals are 500 m. Dredge locations¹² are shown as coloured circles (red, basalts; yellow, gabbro; green, peridotites; blue, others). The inset at centre top displays seismic crustal data versus spreading rate¹⁹. The red stars indicate the values from this study.

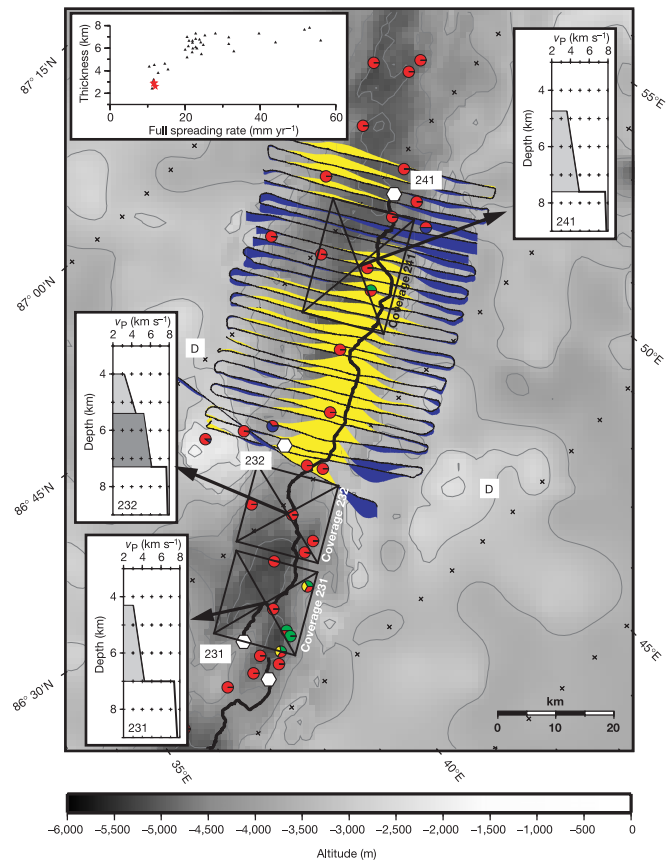


Figure 3 Map of survey area 2, located about 100 km south of area 1. The results of the seismic, magnetic and petrological investigations are shown. For a detailed description see Fig. 2.

The magnetic character changes abruptly at $3^{\circ}30'E$, where the magmatic system apparently collapses and does not generate significant basaltic crust again until basement ridge A at $13^{\circ}E$, after which the magmatic supply is strongly segmented into volcanic basement ridges. The magnetic field in this region is very variable along axis, with the highest amplitudes at the basement ridges. Magma chambers in the rift valley are likely to be associated with these features. This melt focusing appears to be more extreme than observed at other ultraslow-spreading mid-ocean ridges.

The singularity in the magmatic, magnetic and bathymetric expression of the Gakkel ridge at $3^{\circ}30'E$ appears to be a special case, where the mechanisms of melt generation and extraction make an abrupt transition from those more characteristic of spreading rates $>20\text{ mm yr}^{-1}$ to those more typical of ultraslow spreading. However, the existence of elongated off-axis ridges is not restricted to the Gakkel ridge. Along the SWIR (14 mm yr^{-1}) between $15^{\circ}E$ and $25^{\circ}E$, where the ridge is not displaced by large fracture zones, global satellite gravity data show the presence of basement ridges with a spacing of about 100 km. Off-axis, they are visible up to 700 km. This may indicate that off-axis basement ridges are common for spreading velocities $\leq 14\text{ mm yr}^{-1}$. $\square$

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Magnetization distribution in the mixed-phase state of hole-doped manganites

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The effect of ‘colossal magnetoresistance’ (CMR) in hole-doped manganites—an abnormal decrease of resistivity when a magnetic field is applied¹—has attracted significant interest from researchers in the past decade. But the underlying mechanism for the CMR phenomenon is not yet fully understood. It has become clear that a phase-separated state^{2–6}, where magnetic and non-magnetic phases coexist, is important, but the detailed magnetic microstructure of this mixed-phase state is so far unclear. Here we use electron microscopy to study the magnetic microstructure and development of ferromagnetic domains in the mixed-phase state of $\text{La}_{1-x}\text{Sr}_x\text{MnO}_3$ ($x = 0.54, 0.56$). Our measurements show that, in the absence of a magnetic field, the magnetic flux is closed within ferromagnetic regions, indicating a negligible magnetic interaction between separated ferromagnetic domains. However, we also find that the domains start to combine with only very small changes in temperature. We propose that the delicate nature of the magnetic microstructure in the mixed-phase state of hole-doped manganites is responsible for the CMR effect, in which significant conduction paths form between the ferromagnetic domains upon application of a magnetic field.

The framework of double exchange interaction^{7,8} qualitatively explains the phenomenology of the CMR effect in hole-doped manganites, but this mechanism alone is insufficient to quantitatively explain the abnormal transport properties. A prospective picture is the formation of percolative paths for manganese e_g electrons through ferromagnetic (FM) regions⁴—that is, a model highlighting the magnetic microstructure in the mixed-phase state,

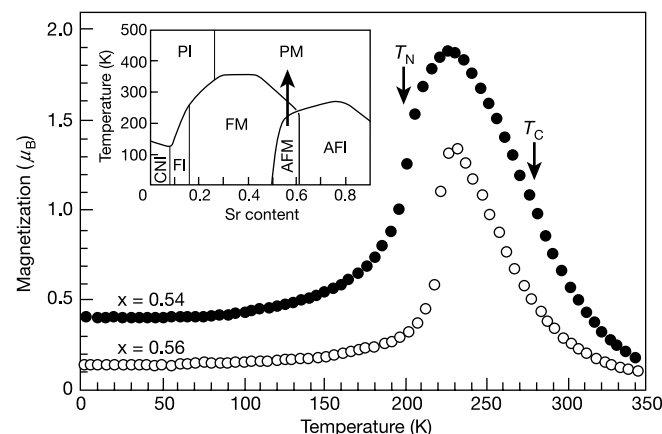


Figure 1 Temperature dependence of magnetization in $\text{La}_{1-x}\text{Sr}_x\text{MnO}_3$ ($x = 0.54, 0.56$). The magnetization was measured on warming in a magnetic field of 1 T. Before the measurement, the specimen was cooled in a magnetic field of 1 T. Inset, the magnetic phase diagram of the same system. See ref. 12 for details. PI, CNI, FI, AFI, T_N and T_C stand for paramagnetic insulator, spin-canted insulator, ferromagnetic insulator, antiferromagnetic insulator, Néel temperature and Curie temperature, respectively.

which may be achieved by applied magnetic fields. In fact, the formation of chained FM regions is supported by recent Monte Carlo simulations^{9,10} and magnetic force microscopy observations¹¹. However, neither the magnetization distribution nor the magnetic correlation among the FM regions have been clearly observed, even for the fundamental condition of no applied magnetic field, although these issues are of vital importance for understanding the peculiar transport properties.

The ceramic specimens $\text{La}_{1-x}\text{Sr}_x\text{MnO}_3$ ($x = 0.54, 0.56$) that were used in the present experiments undergo two successive magnetic phase transformations¹²—the antiferromagnetic (AFM) to FM transformation near 200 K, and the FM to paramagnetic (PM) transformation near 280 K (Fig. 1). Both specimens exhibit similar types of magnetic phase transformations. The AFM to FM transformation is a first-order transformation accompanied by definite change in the lattice parameters^{13–15}. The FM to PM transformation is a second-order-like transformation, exhibiting no appreciable change in the lattice parameters¹⁵. Therefore, two distinct mixed-phase states can be explored using the present specimens.

The magnetic microstructure was observed using a transmission electron microscope (JEM-3000F) in which a magnetically shielded objective lens and a biprism to generate holograms were installed. The residual magnetic field at the specimen position is 0.2 mT. The primary advantage of electron holography^{16,17} is that lines of magnetic flux can be visualized by analysing the phase shift of the electrons that have traversed a specimen. The lines of magnetic flux (strictly speaking, the in-plane component of magnetic flux in the specimen) are displayed as white lines in the reconstructed phase images with Fourier transform of digitized holograms. The phase shift $\phi(x, y)$ is displayed in terms of $\cos\phi(x, y)$, as shown in Figs 2 and 3, so that the density and the direction of lines of magnetic flux correspond to the density and the direction of lines of magnetic flux projected

along the electron beam. However, in practice, the phase shift arises not only from the magnetic field but also from the electric field (due to the mean inner potential) of the specimen. The contribution of the electric field to the observed phase shift is notably serious in specimens with non-uniform thickness. In Fig. 2b, observed slightly above the Néel temperature, the white lines (contour lines) are mostly due to the electric field, where the thickness distribution is strongly outlined, while it is difficult to see the FM region. We have removed the unwanted contribution to the phase from the electric field by subtracting a phase image from the same area at a temperature at which the specimen is in a completely non-magnetic state (Fig. 2a). The contrast seen in the resulting image is then attributed to magnetic fields only (Fig. 2c). Even the newly formed FM region near the arrowhead (Fig. 2c) can be clearly imaged using this approach. The subtraction of the electric field was also performed by Loudon *et al.*¹⁸ to measure the in-plane component of magnetization in $\text{La}_{0.5}\text{Ca}_{0.5}\text{MnO}_3$.

We now focus on the growth process and the magnetization distribution of the FM region that coexists with a non-magnetic phase (presumed to be AFM below 200 K; ref. 15). The volume of the FM region increases by generation of new magnetic domains and their subsequent growth upon heating until the whole area becomes FM (Fig. 2c–h). A noticeable feature is that the magnetic flux is closed within the FM region at every stage of the AFM to FM transformation, resulting in a negligible magnetic interaction between well-separated FM islands without applied magnetic fields. The transformation from FM to AFM took place in the opposite way to that described above. The upper panel of Fig. 2i shows the phase shift along the line X–Y in Fig. 2g. The phase shift increases linearly in the FM region. The spatial distribution of magnetization can be assessed by plotting the differential of the phase shift (Fig. 2i, lower panel)^{16,17}. Both the plateau on the right side, and the steep drop indicated by the arrow, show that the magnetization is uniform over the FM region, but that it abruptly diminishes at the interface with the AFM phase.

The spatial uniformity of magnetization was also checked by measuring the spacing of white lines (l) at five points (red circles in Fig. 2g). The in-plane component of magnetic flux (B) is given by $B = h/etl$, where h is Planck's constant, e the electric charge, and t the specimen thickness at the measured points. t is given by $t = \phi_i / C_E V_0$, where ϕ_i is the phase shift due to the inner potential, C_E a factor related to the wavelength, mass and accelerating voltage of the

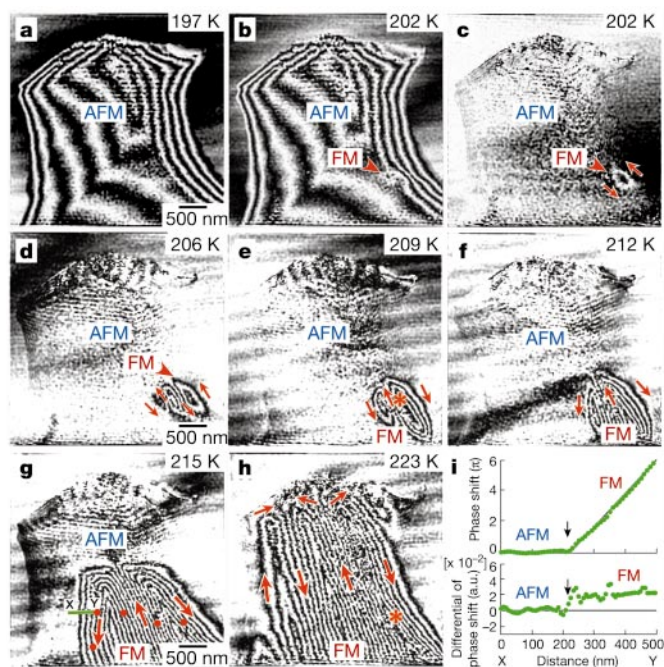


Figure 2 Temperature dependence of the magnetic microstructure in the mixed-phase (ferromagnetic and antiferromagnetic) state in $\text{La}_{0.44}\text{Sr}_{0.56}\text{MnO}_3$. **a, b**, Conventional reconstructed phase images of holograms, where the signal of the electric field is significant. **c–h**, The magnetic information alone, after successful removal of the electric information. Direction of lines of magnetic flux (black-and-white lines in **c–h**) is indicated by red arrows. The upper panel of **i** shows the phase shift along the line X–Y in **g**. The lower panel of **i** provides the differential of the phase shift. The phase shift linearly increases in the FM region: compare with the solid line in the lower panel.

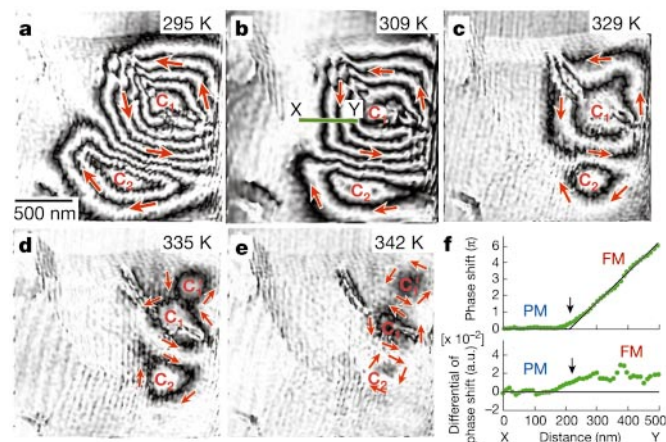


Figure 3 Temperature dependence of the magnetic microstructure in the mixed-phase (ferromagnetic and paramagnetic) state in $\text{La}_{0.46}\text{Sr}_{0.54}\text{MnO}_3$. **a–e**, Reconstructed phase images with the magnetic information alone. Direction of lines of magnetic flux is indicated by red arrows. The upper panel of **f** shows the phase shift along the line X–Y in **b**. The lower panel of **f** provides the differential of the phase shift.

electrons and the speed of light, and V_0 the mean inner potential (calculated to be 23.9 V using scattering factors)^{18,19}. The obtained magnetic flux is converted into magnetization in units of the Bohr magneton, μ_B . The observations were within $(1.7 \pm 0.2) \mu_B$ per Mn. (The observed magnetization is close to the maximum magnetization measured for the bulk specimen of $\text{La}_{0.44}\text{Sr}_{0.56}\text{MnO}_3$ ($1.3 \mu_B$, Fig. 1), but smaller than the theoretical value ($3.4 \mu_B$) expected for the fully aligned magnetic moments on each atom. A possible explanation is that the magnetic moments are canted. The surface layer that may be damaged by ion-milling will also reduce the observable magnetization.) We see that the spacing of the white lines is almost unchanged through the transformation, that is, the FM island has strong magnetization from the beginning of its formation in the AFM phase. In fact, the in-plane component of magnetization at the position shown by the asterisk in Fig. 2e and h was $1.6 \mu_B$ for both 209 K and 223 K.

In contrast, the spontaneous magnetization becomes gradually smaller during the FM to PM transformation as the FM regions shrink. This can be seen by the increased spacing of the contour lines as the specimen is heated (Fig. 3a–e). This observation distinguishes the second-order-like FM to PM transformation from the first-order AFM to FM transformation. An interesting feature is that the closure domain (C_1 , Fig. 3a) splits into two parts (C_1 and C_1' , Fig. 3e). In both of these domains, the magnetic flux circulates in the same direction. As a result of the splitting and shrinkage, the mixed-phase state is also achieved in the second-order-like FM to PM transformation when observed microscopically. The PM to FM reverse transformation (not shown) proceeded via the growth of the closure magnetic domains and/or their merging. The upper panel of Fig. 3f shows the phase shift along the line X–Y in Fig. 3b. The lower panel provides the differential of the phase shift. The differential gradually increases toward the FM region, in contrast to the step-like increase in Fig. 2i; that is, the phase boundary is obscure between the FM and PM phases, while it is clear between the FM and AFM phases. Again, the magnetic flux is closed within the FM

region at any stage of the coexistence with the PM phase, resulting in a negligible magnetic interaction between well-separated FM islands in the absence of magnetic fields.

The question then arises as to how delicate is the magnetic microstructure in the mixed-phase state. An essential feature was derived from Lorentz microscopy observations, which visualize magnetic domain walls as bright or dark lines, as indicated by the black arrows in Fig. 4. Two FM regions are observed in Fig. 4a, where the magnetic flux was confirmed to be closed within each FM region so that no stray field was produced, being similar to the results in Fig. 2. When the electron beam current is increased from 0.13 mA cm^{-2} (Fig. 4a) to 0.17 mA cm^{-2} (Fig. 4b), the separate FM regions are combined into a larger one while the volume of the AFM phase is reduced. (The beam current given here represents the value at the specimen position.) The magnetic domain walls are also shifted slightly. The large FM region was again separated into the smaller ones by the subsequent reduction of beam current. This microstructural change is presumably caused by a small increase (or decrease) of temperature due to the illumination change, although the thermocouple placed near the specimen could not detect such a local change in temperature. By contrast, when the specimen is in the single-phase (FM) state, increase of the beam current does not modify the magnetic microstructure (Fig. 4c and d)—for example, the domain walls never move with the beam heating. The dramatic change in the microstructure in the mixed-phase state is due to the coexistence of phases in thermodynamic equilibrium: only a small change in temperature assists the growth of the energetically favoured phase. It is quite reasonable to consider that application of magnetic fields and/or stress plays a similar role in modifying the magnetic microstructure. If a metallic FM phase coexists with an insulating non-magnetic phase, the application of magnetic field may connect the separated FM regions in a similar fashion to that shown in Fig. 4a and b. This delicacy of the magnetic microstructure in the mixed-phase state is believed to be essential to the realization of significant conduction paths for e_g electrons upon the application of magnetic fields.

Recent progress in electron microscopy has enabled us to observe the magnetization distribution of manganites in detail. Loudon *et al.*¹⁸ performed electron holography studies of $\text{La}_{0.5}\text{Ca}_{0.5}\text{MnO}_3$. They discovered a new phase—the ‘charge-ordered ferromagnetic state’—that is achieved at low temperature. The present holography studies have disclosed the temperature dependence of the magnetic microstructure: the spread of magnetic flux has been clearly mapped. Moreover, the present observations have revealed that there is only a negligible magnetic interaction between well-separated FM islands, but that these islands are believed to interact with each other when a magnetic field is applied. We believe that the observations reported here will help the understanding of the physical processes underlying CMR phenomena. □

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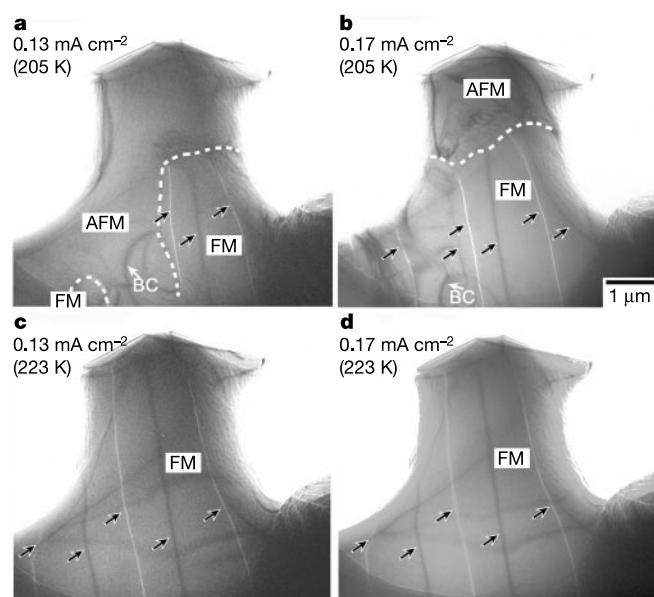


Figure 4 Effect of beam heating on the magnetic microstructure in the mixed-phase state and the single-phase state in $\text{La}_{0.44}\text{Sr}_{0.56}\text{MnO}_3$. **a, b**, The merging of two separated ferromagnetic islands with a slight change in illumination for the mixed-phase state (ferromagnetic and antiferromagnetic). **c, d**, The results for the single-phase state (ferromagnetic), where no appreciable change is observed. The temperature change was too small and localized to allow detection by the thermocouple that was placed near the specimen (no direct connection to the specimen). BC, bend contour arising from the diffraction effect.

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Three-dimensional binary superlattices of magnetic nanocrystals and semiconductor quantum dots

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Recent advances in strategies for synthesizing nanoparticles—such as semiconductor quantum dots¹, magnets and noble-metal clusters²—have enabled the precise control of composition, size, shape³, crystal structure⁴, and surface chemistry. The distinct properties of the resulting nanometre-scale building blocks can be harnessed in assemblies with new collective properties^{2,5,6}, which can be further engineered by controlling interparticle spacing and by material processing. Our study is motivated by the emerging concept of metamaterials⁷—materials with properties arising from the controlled interaction of the different nanocrystals in an assembly. Previous multi-component nanocrystal assemblies have usually resulted in amorphous or short-range-ordered materials^{8,9} because of non-directional forces or insufficient mobility during assembly^{10–14}. Here we report the self-assembly of PbSe semiconductor quantum dots and Fe_2O_3 magnetic nanocrystals into precisely ordered three-dimensional superlattices. The use of specific size ratios directs the assembly of the magnetic and semiconducting nanoparticles into AB_{13} or AB_2 superlattices with potentially tunable optical and magnetic properties. This synthesis concept could ultimately enable the fine-tuning of material responses to magnetic, electrical, optical and mechanical stimuli⁶.

Nanocrystal (NC) superlattices have attracted increasing attention since they were first reported⁸ in 1989. Studies of colloidal microspheres (natural and synthetic) have shown that mixtures of

such spheres with selected size ratios can co-crystallize into binary AB , AB_2 , AB_5 or AB_{13} colloidal crystals^{15–18}. Further examples of binary nanocrystal assemblies can be found in refs 5 and 19, reporting ordered monolayers displaying the binary AB_2 or AB composition, or a trilayer consistent with the AB_5 structure. Here we present methods that extend the assembly to three dimensions and longer length scales, and use NCs with independently tunable optical and magnetic properties^{20,21}. In our model system we co-assemble PbSe semiconductor quantum dots and $\gamma\text{-Fe}_2\text{O}_3$ magnetic nanocrystals to produce binary superlattices. The PbSe NCs display highly size-dependent near-infrared optical absorption (Supplementary Fig. 3) and emission with high quantum yield due to quantum confinement²¹. Bulk $\gamma\text{-Fe}_2\text{O}_3$ is reported²² to be transparent at wavelengths longer than 650 nm. The 11-nm $\gamma\text{-Fe}_2\text{O}_3$ particles used here are superparamagnetic at room temperature²⁰. The average magnetization of the NCs responds rapidly to external magnetic fields.

The $\gamma\text{-Fe}_2\text{O}_3$ NCs are synthesized by decomposition of iron pentacarbonyl in trioctylamine (boiling point 365 °C) with oleic acid as surfactant⁸, and a subsequent oxidation with trimethylamine N-oxide following a general 'one-pot' synthesis²⁰. The narrow distribution of sizes ($\sigma = 5\%$) of the resulting particles enables the formation of close-packed superlattices during slow evaporation of solvent from the dispersions. X-ray powder diffraction patterns confirm the $\gamma\text{-Fe}_2\text{O}_3$ structure. At higher resolution weak Bragg reflections are visible (Supplementary Fig. 6), characteristic of a tetragonal superstructure^{22,23}. Optical absorption measurements of particle dispersions (Supplementary Fig. 3) show a strong absorption in the visible and a weaker absorption in the near-infrared at a wavelength of 1,400 nm. The infrared absorption can be attributed to a charge-transfer transition between Fe^{3+} and Fe^{2+} (ref. 22). This is due to incomplete oxidation during the synthesis. This absorption is strongly reduced after further oxidation at 200 °C in air or in solution (Supplementary Fig. 3c). As derived from X-ray powder diffraction measurements, the tetragonal superstructure is maintained after complete oxidation as well as the NC diameter. The absorption of the individual components is displayed in Supplementary Fig. 3a, b. When assembled into a binary superlattice, the PbSe absorption is observed along with the background absorption of the $\gamma\text{-Fe}_2\text{O}_3$ NCs.

Co-crystallized assemblies of PbSe and $\gamma\text{-Fe}_2\text{O}_3$ nanocrystals were prepared with guidance from established colloidal chemistry^{15–18}; we used systematic variation of NC sizes, concentrations and deposition rate to optimize assembly conditions. Size ratios between PbSe and $\gamma\text{-Fe}_2\text{O}_3$ particles were varied between 0.4 and 0.7, while the relative concentrations were adjusted around the optimal composition of the target structure (for example, 1:13 for AB_{13}). We determined the NC concentrations of the stock dispersions by drying, weighing and using elemental analysis to correct for extraneous organics, and then correlating concentrations with the absorption of the dispersions. Hexane, octane and dibutyl ether were tested as solvents in the drying process, and the temperature of evaporation was increased from room temperature to 60 °C. The deposition rate is adjusted by controlling the solvent vapour pressure above samples during drying. The best binary superlattices are generated by evaporation of dibutyl ether at 60 °C over eight hours from diluted mixed dispersions containing a tenfold surplus of 6-nm PbSe particles for each 11-nm $\gamma\text{-Fe}_2\text{O}_3$ NC.

Transmission electron microscope (TEM) images of the binary superlattices are shown in Fig. 1c, d, f, g, Fig. 2c, d, g, h, k, l, and Supplementary Fig. 4d, e. The observed projections of the three-dimensional assembly can be assigned to crystal structures known from the intermetallic compounds NaZn_{13} , AlB_2 and CaCu_5 . A unit cell of the AB_{13} compound NaZn_{13} (space group (SG) 226) is shown in Fig. 1b. The larger $\gamma\text{-Fe}_2\text{O}_3$ NCs form a cubic framework, with an icosahedron of smaller PbSe NCs in the middle (Fig. 1a). The thirteenth PbSe NC is located in the centre of the icosahedron.

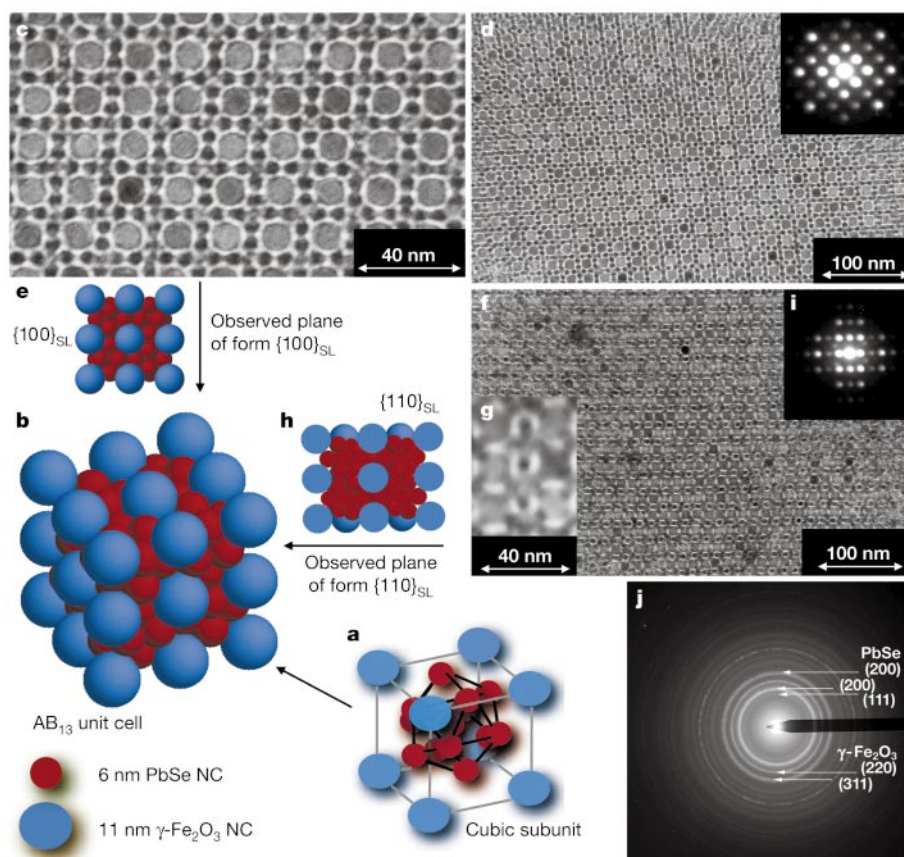


Figure 1 TEM micrographs and sketches of AB_{13} superlattices (isostructural with intermetallic phase $NaZn_{13}$, SG 226) of 11-nm $\gamma\text{-Fe}_2\text{O}_3$ and 6-nm PbSe NCs. **a**, Cubic subunit of the AB_{13} unit cell. **b**, AB_{13} unit cell built up of eight cubic subunits. **c**, Projection of a $\{100\}_{SL}$ plane at high magnification. **d**, As **c** but at low magnification; inset: small-angle electron diffraction pattern from a corresponding $6\text{-}\mu\text{m}^2$ area. **e**, Depiction of a

$\{100\}$ plane. **f**, Projection of a $\{110\}_{SL}$ plane. **g**, As **f** but at high magnification. **h**, Depiction of the projection of the $\{110\}$ plane. **i**, Small-angle electron diffraction pattern from a $6\text{-}\mu\text{m}^2$ $\{110\}_{SL}$ area. **j**, Wide-angle electron diffraction pattern of an AB_{13} -superlattice (SAED of a $6\text{-}\mu\text{m}^2$ area) with indexing of the main diffraction rings for PbSe and $\gamma\text{-Fe}_2\text{O}_3$ (maghemite).

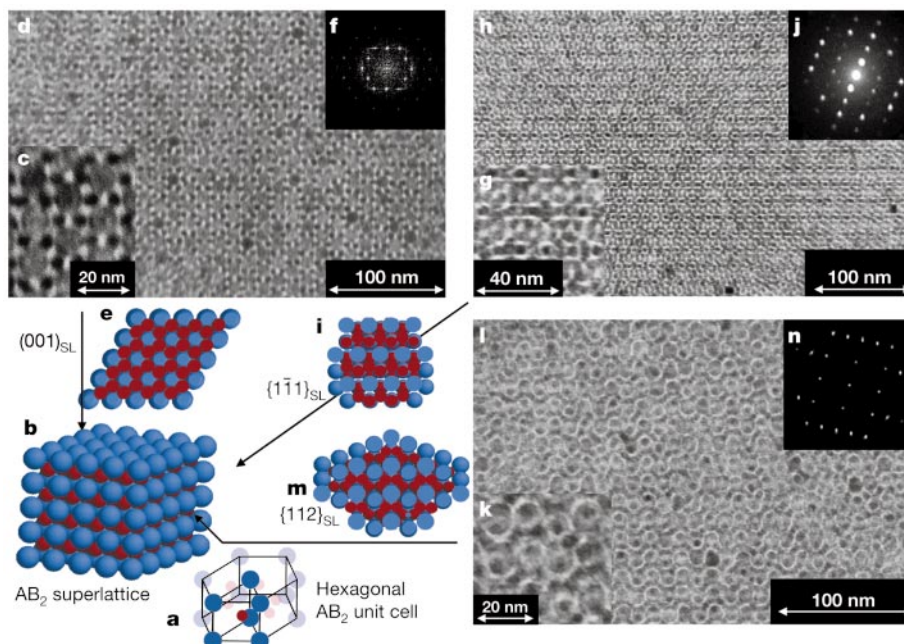


Figure 2 TEM micrographs and sketches of AB_2 superlattices (isostructural with intermetallic phase AlB_2 , SG 191) of 11-nm $\gamma\text{-Fe}_2\text{O}_3$ and 6-nm PbSe NCs. **a**, Hexagonal unit cell of the AB_2 lattice. **b**, AB_2 superlattice built up of alternating layers of large and small particles. **c**, Projection of the $\{001\}_{SL}$ plane. **d**, As **c** but at lower magnification. **e**, Depiction of the $\{001\}$ plane. **f**, FFT of **d**. **g**, Projection of a $\{111\}_{SL}$ plane. **h**, As **g** but at lower magnification. **i**, Depiction of the projection of a $\{111\}_{SL}$ plane. **j**, Small-angle electron diffraction pattern from a $6\text{-}\mu\text{m}^2$ SL area. **k**, Projection of the $\{112\}_{SL}$ plane. **l**, As **k** but at lower magnification. **m**, Depiction of the projection of a $\{112\}_{SL}$ plane. **n**, FFT of **l**.

e, Depiction of the $\{001\}$ plane. **f**, FFT of **d**. **g**, Projection of a $\{111\}_{SL}$ plane. **h**, As **g** but at lower magnification. **i**, Depiction of the projection of a $\{111\}_{SL}$ plane. **j**, Small-angle electron diffraction pattern from a $6\text{-}\mu\text{m}^2$ SL area. **k**, Projection of the $\{112\}_{SL}$ plane. **l**, As **k** but at lower magnification. **m**, Depiction of the projection of a $\{112\}_{SL}$ plane. **n**, FFT of **l**.

Adjacent icosahedrons are twisted by 90° , leading to a unit cell composed of eight cubes. The TEM images in Fig. 1c, d show one of the $\{100\}_{\text{SL}}$ planes of a superlattice with each $\gamma\text{-Fe}_2\text{O}_3$ NC spaced with eight small PbSe NCs to form a square array (Fig. 1e). A further plane of the AB_{13} superlattice (Fig. 1f–h) consists of big particles forming rectangles with a ring of small particles in the middle (interference due to particles at different heights leads to a ring-like contrast pattern) surrounding an additional large particle (the icosahedron is cut through the middle). This projection is characteristic of the $\{110\}$ planes of the AB_{13} crystal. The average cubic unit cell, with a formal composition of 8 $\gamma\text{-Fe}_2\text{O}_3$ and 104 PbSe nanocrystals, has an edge length of about 36 nm and incorporates about 4.5×10^6 atoms.

The AB_2 superlattice is based on a hexagonal unit cell (SG 191, Fig. 2a). The structure consists of parallel layers of hexagonally ordered large particles with small particles in every trigonal prismatic interstice (Fig. 2b). Figure 2d and c shows the $(001)_{\text{SL}}$ plane of the AB_2 structure. The particles appear to touch each other in the two-dimensional projection, but the small particles in fact form a second layer above the hexagonal layer of iron oxide nanocrystals, as confirmed by stepping the depth of focus. Each one of the PbSe nanocrystals is occupying the gap between three touching large particles. Figure 2g and h display a projection of the $\{111\}_{\text{SL}}$ planes of the AB_2 structure. Parallel rows of large particles form an array of parallelograms or alternative distorted hexagonal packing. This geometry is confirmed in the selected-area electron diffraction (SAED) pattern (Fig. 2j). The visibility of individual particles and their real dimensions are distorted by interference between different layers. The $\{112\}$ planes project an image with wave-like alternating layers of large and small particles (Fig. 2l, k). The symmetry of each layer is confirmed by fast Fourier transform (FFT) of the field of view and a small-angle electron diffraction pattern from an area of several square micrometres. The lattice constants can be measured from both the real-space TEM images and the diffraction patterns to be 12–13 nm for a and 15 nm for c . The intermediate layer of small PbSe particles causes the elongation in the c direction.

The third observed superlattice (Supplementary Fig. 4d, e) is isostructural with CaCu_5 (SG 191)^{18,19}. The two-dimensional structure (incorporating three layers of small particles) has been obtained from 11-nm $\gamma\text{-Fe}_2\text{O}_3$ NCs and 6.3-nm PbSe NCs. This preliminary observation shows small regions of ordering (up to $0.16 \mu\text{m}^2$) with numerous point defects. The structure and the unit cell are depicted in Supplementary Fig. 4a, b. Large particles in a hexagonal array are separated by small particles occupying the trigonal interstices. Parallel layers of this arrangement are separated by a hexagonal net of small particles. Alternatively, the structure can be described as a trigonal prism of large particles that has a small particle at the centre of each face. Only the (001) plane has been detected. An average unit cell length a_{SL} of 17 nm is measured.

In these samples, single domains of the AB_2 and AB_{13} superlattices range from 0.16 to $2 \mu\text{m}^2$ in area. Whereas short-range order is routinely observed as the size ratio approaches values between 0.55 and 0.58, long-range order requires tight control of particle size, size distribution and size ratio, as well as slow deposition. Samples typically contain binary superlattices separated by narrow grain boundaries coexisting with phase-separated superlattices of pure small or large NCs (Supplementary Fig. 5). Local variation in concentrations or non-uniform drying rates may be important during superlattice growth. Thicker regions tend to favour binary superlattices, while in areas of lower coverage more phase-separated regions are observed²⁴. Transparency limits the thickest superlattice imaged to between four to six layers, although with longer integration times electron diffraction confirms ordering in samples as thick as approximately 15 layers.

The best AB_{13} and AB_2 superlattices in our studies were formed by systems with a diameter ratio $d_{\text{PbSe}}/d_{\gamma\text{-Fe}_2\text{O}_3}$ of 0.55. The shortest distance between $\gamma\text{-Fe}_2\text{O}_3$ NCs is about 1.8 nm, governed by the

surfactant oleic acid (measured by TEM). The effective particle diameter²⁵ is given by $d_{\text{NC}} + 1.8 \text{ nm}$, resulting in an effective particle size ratio of 0.58 that is within the calculated range for assembly of non-interacting spheres into AB_2 or AB_{13} colloidal crystals^{15,26}. These binary colloidal crystals have densities similar to, or slightly higher than, phase-separated superlattices. Better crystal quality was correlated with higher deposition temperatures²⁷ and slower drying times²⁸. This may be due to the benefits of greater particle mobility allowing the formation of superlattice structures. Our optimal results were achieved by slow deposition at 60°C from dibutyl ether dispersion. The AB_5 (CaCu_5) structure shown in Supplementary Fig. 4 was obtained by mixing particles with a slightly increased diameter ratio. The effective diameter ratio of 0.63 is slightly below the previously observed ratios for the AB_5 structure in colloidal chemistry¹⁸.

SAED patterns of colloidal crystals (Fig. 1j) reveal the internal structure of the $\gamma\text{-Fe}_2\text{O}_3$ and PbSe NCs comprising the superlattices. The uniform rings indicate that the orientation of the individual NCs is independent of the superlattice axis, although the positions of the NCs are rigorously controlled. Orientational ordering of NCs has been observed in other superlattices of highly faceted NCs²⁹.

The binary NC superlattices that we report here consist of two sets of NCs, each with size-tunable properties, and each with the potential to interact/couple with neighbouring particles less than 2 nm away. There are a growing number of monodisperse NC systems available in the research community, which could enable the development of a large family of binary NC materials. These multi-component NC assemblies may in turn allow engineering of more complex materials from which new properties may emerge. □

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Modulated structure of solid iodine during its molecular dissociation under high pressure

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The application of pressure to solid iodine forces the molecules in the crystal to approach each other until intermolecular distances become comparable to the bond length of iodine; at this point, the molecules lose their identity and are essentially dissociated. According to room-temperature X-ray diffraction studies¹, this process involves direct dissociation of iodine molecules at about 21 GPa, whereas spectroscopic observations^{2,3} have identified intermediate molecular phases at pressures ranging from 15 to 30 GPa. Here we present quasi-hydrostatic powder X-ray diffraction measurements that clearly reveal an intermediate phase during the pressure-induced dissociation of solid iodine. We find that, similar to the behaviour seen in uranium⁴, the structure of this intermediate phase is incommensurately modulated, with the nearest interatomic distances continuously distributed over the range 2.86–3.11 Å. The shortest of these interatomic distances falls between the bond length of iodine in the molecular crystal (2.75 Å) and the nearest interatomic distance in the fully dissociated monatomic crystal (2.89 Å), implying that the intermediate phase is a transient state during molecular dissociation. We expect that further measurements at different temperatures will help to elucidate the origin and stability of the incommensurate structure, which might lead to a better understanding of the molecular-level mechanism of the pressure-induced dissociation seen here and in the molecular crystals of hydrogen⁵, oxygen⁶ and nitrogen⁷.

Solid iodine forms an orthorhombic molecular crystal made up

of four I₂ molecules in a unit cell with space group *Cmca* (phase I). Application of pressure forces the molecules to approach each other^{8,9}, resulting in an insulator-to-metal transition near 16 GPa (refs 10, 11). The metallization accompanies no structural change, and iodine continuously transforms into a molecular metal. At higher pressures, the intermolecular distances become comparable to the intramolecular distance, where molecules lose identity and dissociate. This pressure-induced molecular dissociation was first observed in iodine at 21 GPa by powder X-ray diffraction experiments¹. The crystal structure of the monatomic phase was determined to be body-centred orthorhombic, with two atoms in a unit cell with space group *Immm* (phase II)¹. Phase II further transforms to a body-centred-tetragonal phase (phase III) at 43 GPa (ref. 12), and to a face-centred-cubic phase (phase IV) at 55 GPa (ref. 13). Phase IV persists to at least 276 GPa (ref. 14). Whereas X-ray diffraction experiments indicated direct dissociation of iodine molecules, Mössbauer² and Raman³ experiments suggested the existence of intermediate molecular phases in the pressure range of 15–30 GPa. In the present study, we aimed at reconciling the discrepancy by using a He pressure-transmitting medium, which provides excellent quasi-hydrostatic conditions¹⁵. In contrast, all previous high-pressure experiments—including those using X-ray¹, Mössbauer² and Raman³ techniques—lacked a pressure-transmitting medium, and thus involved non-hydrostatic conditions.

X-ray patterns taken at low pressures correspond to an orthorhombic *Cmca* structure, with new diffraction peaks indicative of a phase transition first detected at 23.2 GPa. This pressure is higher than that reported in the early investigation¹, which we attribute to the better quasi-hydrostaticity in the present experiment. The diffraction peaks of the low-pressure phase disappeared at 24.6 GPa, but the diffraction pattern was different from that

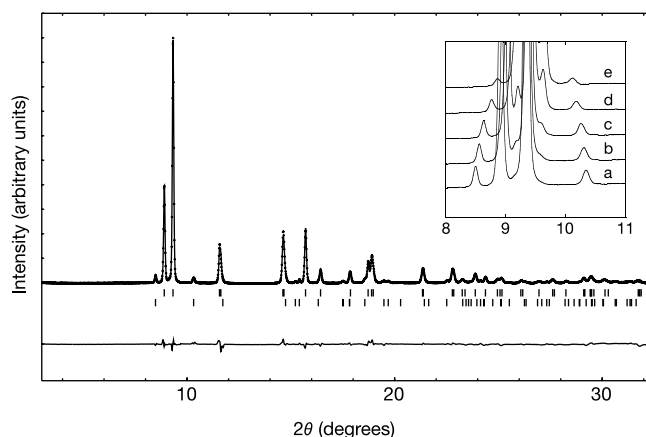


Figure 1 Powder X-ray diffraction pattern of the intermediate phase V of iodine. Data are shown for 24.6 GPa (small crosses) together with the Rietveld refinement (line). The background is subtracted. Tick marks just below the pattern indicate the positions of the main reflections of the basic f.c.c. cell. Lower tick marks indicate the positions of satellite reflections. The lowest curve shows the difference between the observed and calculated diffraction intensities. The inset shows the change of the positions of the satellite reflections with pressure (GPa): trace a, 24.6; b, 25.5; c, 26.8; d, 28.4; and e, 29.5. Peaks of the monatomic phase II appear above 25.5 GPa near $2\theta = 9.3^\circ$ and 9.7° . High-pressure experiments were performed with a diamond-anvil cell (DAC) at room temperature. Iodine with a stated purity of 99.999% was ground under an Ar atmosphere. The powdered sample was placed into a hole in a Re gasket, together with ruby spheres for the pressure measurement. The rest of the gasket hole was filled with He (ref. 22). Pressure was determined using the ruby pressure scale²³. Diffraction patterns were taken on beamline BL-13A of the Photon Factory. The incident X-rays were monochromatized to a wavelength of 0.4264 Å, and collimated to a diameter of 50 μm. We used an imaging plate²⁴ and the pattern integration software pip (F.H., unpublished work). A diamond-backing plate²⁵ was used as a window in the DAC to collect full diffraction rings.

expected for phase II. Upon further increase of the pressure to 25.5 GPa, another set of peaks appeared, which could be assigned to phase II. The pure pattern of phase II was obtained at 30.4 GPa and above. These observations indicate the existence of an intermediate phase between the molecular phase I and the monatomic phase II. We call this intermediate phase 'phase V'. The change in the X-ray diffraction patterns was fully reversible when we released the pressure. Two independent experimental runs gave identical results.

Figure 1 shows the diffraction pattern of phase V at 24.6 GPa. The strong peaks (upper tick marks) can be indexed to a face-centred orthorhombic (f.c.o.) lattice with four atoms in a unit cell. However, a number of weak peaks (lower tick marks) remain unindexed. The behaviour of the weak peaks is unusual; some of them shift to lower scattering angles with increasing pressure, indicating an increase of the interplanar spacings with pressure. Careful inspection of the

patterns reveals that the unusual peaks are accompanied by counterparts that move normally with pressure, that is, to higher scattering angles (Fig. 1 inset). This strongly suggests that the unindexed peaks are satellite reflections of the main peaks. On this assumption, we analysed the pattern with the program PREMOS¹⁶; this program analyses powder diffraction data of modulated structures with the Rietveld method. We have considered first-order modulation, as only the first-order satellite reflections are observed. Assumption of higher-order modulations always results in nearly zero amplitude for them. After several trials, we found that the modulation wave propagates along the *a* axis. In terms of the superspace-group representation, this structure is written as $Fmm2(\alpha 00)0s0$, which is equivalent to $F2mm(00\gamma)0s0$ in the literature¹⁷. Iodine atoms occupy the $4a$ position $(0, 0, z)$ with $z = 0$. A variable structural parameter is the sine term of the Fourier amplitude $B_1(y)$ constrained by the symmetry. The result of the refinement is excellent, with the reliability factor $R_{wp} = 2.3\%$. The lattice parameters (in Å) for the basic f.c.o. cell are $a = 4.2280(6)$, $b = 4.2039(6)$ and $c = 5.4868(8)$. The modulation vector is $\mathbf{k} = (0.257, 0, 0)$ with $B_1(y) = 0.053(2)$.

The diffraction pattern can also be analysed as a modulation of a face-centred tetragonal (equivalent to body-centred tetragonal) lattice, since the difference in the length of the *a* and *b* axis of the f.c.o. cell is small. The corresponding superspace group for the body-centred tetragonal cell is $I4mm(-\alpha\alpha 0, \alpha\alpha 0)0gg$ with lattice parameters (in Å) of $a_T = b_T = 2.9811$ and $c = 5.4868$. The Rietveld refinement yields $R_{wp} = 3.8\%$, which is larger than the R_{wp} value obtained for the f.c.o. cell. We therefore adopt the f.c.o. cell to describe the modulated structure, although we cannot rule out the face-centred tetragonal cell.

Figure 2 compares the crystal structures of phases I, V and II. Iodine atoms form a two-dimensional network in the *a*–*b* plane in phase V. The modulation wave is transverse, which periodically shifts the atoms in the direction parallel to the *b* axis. The wavelength $1/k$ of the modulation wave is not a multiple of the length of the *a* axis, making the structure incommensurate. As a result of the modulation and incommensuration, the atomic positional coordinate *y* varies along the *a* axis, giving rise to a continuous distribution of the near interatomic distances. There are

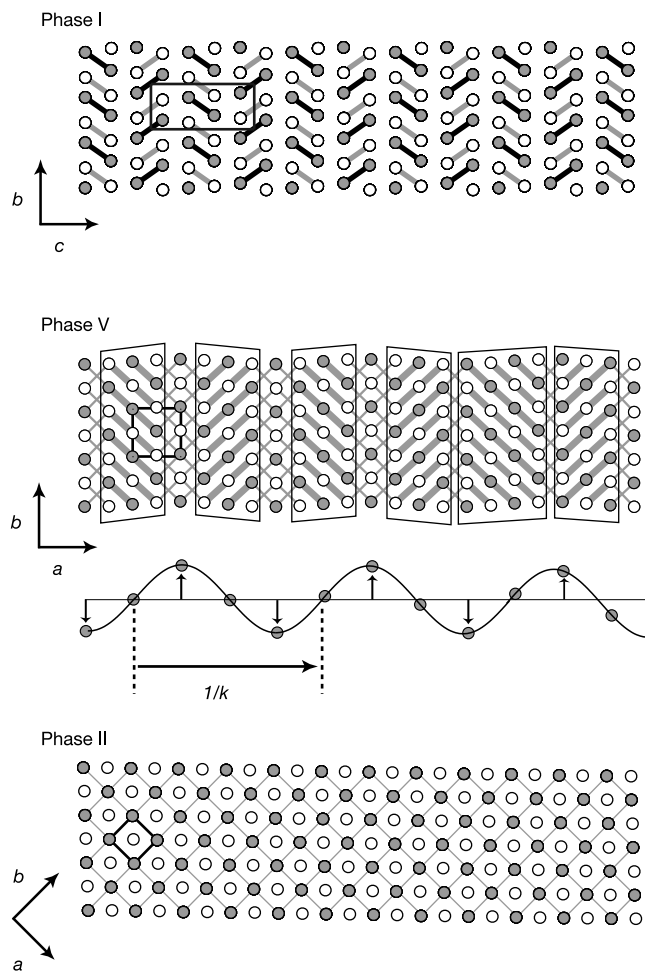


Figure 2 Crystal structure of iodine for phases I (19.1 GPa), V (24.6 GPa) and II (30.4 GPa). The figure shows the projection of atoms onto the planes indicated. Filled and open circles indicate respectively the atoms lying in the plane and in the adjacent plane halfway below. The unit cells for each phase are indicated by small rectangles. Phase I (top panel) consists of I_2 molecules, shown by thick lines. Phase V (middle panel) has no molecular units. The interatomic distances are distributed in the range 2.86–3.11 Å. In order to distinguish the small difference in the distances, we connect atoms at a distance 2.86–2.92 Å by thick lines, and those at 2.92–3.05 Å by thin lines. Atoms at a distance 3.05–3.11 Å are not connected for the sake of clarity. Three- or four-atom chains appear, which form domains as indicated by parallelograms. Below the crystal structure is shown the modulation wave, whose amplitude is exaggerated so as to clarify the modulation. The positions of atoms progressively shift on the wave, showing the incommensurate nature. Phase II (bottom panel) has a simple structure described by the body-centred orthorhombic lattice. The choice of the axes is different from that in ref. 1.

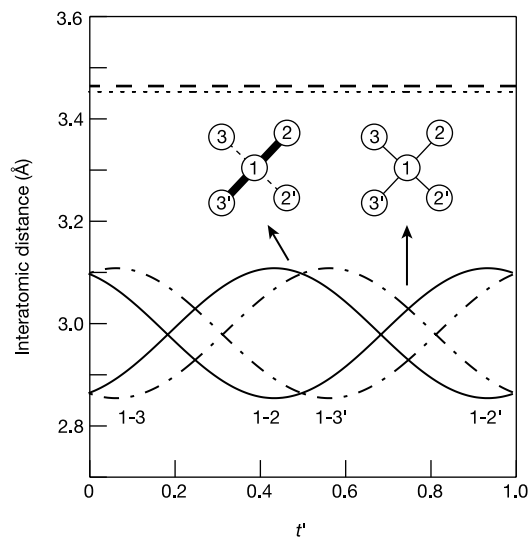


Figure 3 Variation of the interatomic distances of phase V at 24.6 GPa as a function of supplementary coordinate t' . Here $t' = t - \mathbf{k} \cdot \mathbf{x}$, where t is the phase of the modulation wave, and $\mathbf{k}$ and $\mathbf{x}$ are the modulation wavevector and the atom position, respectively¹⁶. Two representative atomic arrangements are illustrated. The distances near 3.46 Å are those between atoms belonging to two adjacent planes.

domains where atoms gather closer. Notice that the repeat cycle of the domains along the a axis lacks periodicity. The magnitude of the modulation vector k decreases from 0.26 at 24.6 GPa to 0.17 at 29.5 GPa, as is anticipated from the convergence of the satellite reflections to the main reflection (Fig. 1 inset). The modulation amplitude remains nearly constant with pressure.

It is worth noting that the structure virtually becomes a superstructure of the basic f.c.c. cell having the a axis four times larger than the basic one, when the modulation vector takes a value 0.25 at about 25 GPa. We found no evidence for an incommensurate-commensurate transition at this pressure, as the modulation vector steadily decreases with pressure through this point. Temperature-dependent high-pressure experiments would be very interesting in this regard. Phase I coexists with phase V over a pressure range of 23–24 GPa, while phase V coexists with phase II over a larger pressure range, 25–30 GPa. The volume changes at the I–V and the V–II transitions are 2.0% and 0.2%, respectively, which show no pressure dependence. These observations indicate that the two transitions are of first order. We also note that the lattice parameters of phases I and II in the present experiments are consistent with those obtained in the experiments without using a pressure medium^{1,8,9}. We therefore rule out the possibility of the diffusion of helium into the iodine lattice. Although weak, some of the satellite reflections of phase V are also recognized in the X-ray patterns taken without a pressure medium (F. H., unpublished results).

Figure 3 shows the variations of the interatomic distances of phase V as a function of supplementary coordinate t' (ref. 16). The four near-neighbour distances vary with correlations. Two extreme cases of the atomic arrangement are shown in the figure. In the first case near $t' \approx 0.5$, the central atom has two near neighbours aligned

in a line, accompanied by two next-nearest neighbours located in the perpendicular directions. This type of arrangement can be seen in the domains shown in Fig. 2. In the second case near $t' \approx 0.75$, the four neighbours are located at nearly equal distances. This is the arrangement found in the region between two adjacent domains in Fig. 2. The general atomic arrangement lies between the two extremes mentioned. The maximum difference among the four near-neighbour distances is 3–8%. Figure 4 compares the near interatomic distances for phases I, V and II. The nearest distance (2.86 Å) of phase V is definitely larger than the bond length of I_2 molecule (2.75 Å) in phase I, clearly showing that no diatomic molecules exist in phase V. It is, however, difficult to decide whether phase V has molecular or monatomic character. As we have seen in Figs 2 and 3, linear chains of atoms exist in some places in the crystal, but not in other places. The distribution of interatomic distances is exactly intermediate between those for phases I and II (Fig. 4). From these considerations, we conclude that phase V is an intermediate state between a molecular and a monatomic state.

Phase V may have relevance to one of the phases proposed on the basis of Mössbauer data². The crystal structure proposed in that work consisted of two-dimensional network of I_2 molecules, which mimics the present incommensurate structure. An important difference is that the authors of ref. 2 considered the phase to have molecular character, while the present phase V lies on the boundary between the molecular and monatomic states. We interpret the formation of phase V as a result of iodine molecules first dissociating at the I–V transition, with the dissociated atoms not immediately forming a simple commensurate structure, but developing small domains with variable interatomic distances. This behaviour would give rise to a modulation wave ordering the overall atomic arrangement across the domains. Fluctuations between molecular and monatomic states could thus account for the modulation wave apparent in our data, but further experimental and theoretical studies are needed to substantiate this interpretation.

The existence of an incommensurate structure or a charge density distortion wave in the vicinity of molecular dissociation has been theoretically discussed^{9,18}. Incommensurate structures are known for some high-pressure phases of the elements. The high-pressure phase IV of barium, for example, takes a self-hosting incommensurate structure, where the guest structure is incommensurate to the host structure¹⁹. The structure of iodine V is obviously different from barium IV, as it has no host–guest arrangement. Iodine V can best be compared with the α -phase of uranium below 37 K, a well-known example of an incommensurately modulated structure⁴. We expect that other diatomic molecular crystals may have similar modulated phases when they dissociate under high pressure. Because of its close similarity to iodine²⁰, bromine may have such a modulated phase near the molecular dissociation at 80 GPa (ref. 21). Although a number of materials are known to have incommensurate structures, iodine V is unique in the sense that it is intimately related to the dissociation process of the molecules, with the present study offering a structural model for first-principles calculations and molecular dynamics simulations aimed at elucidating pressure-induced molecular dissociation. □

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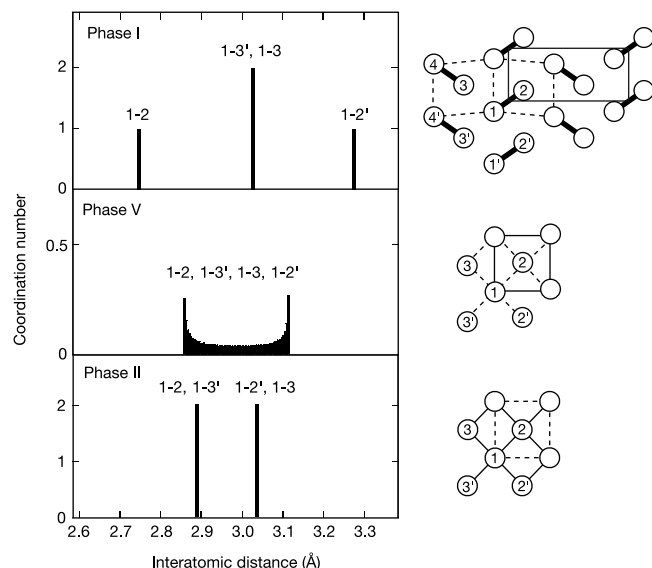


Figure 4 Distribution of the near interatomic distances for phases I (19.1 GPa), V (24.6 GPa) and II (30.4 GPa). The arrangement of atoms in each phase is shown on the right, which depicts atoms on the initial molecular plane (the b - c plane of phase I). The solid and broken lines indicate respectively the unit cell and the relationship between the different unit cells. The four nearest-neighbour distances are continuously distributed in phase V. The diagram for phase V is hence obtained by calculating the interatomic distances for 2,500 unit cells along the a axis, and taking the histogram with a step of 0.004 Å. The vertical scale of the diagram is normalized so that the integrated frequency becomes equal to four, as in phases I and II. The diagram is equivalent to the density of states of Fig. 3. The lattice parameters and atomic coordinates for phases I and II are from the present experiments: $a = 5.8287(10)$ Å, $b = 3.9671(7)$ Å, $c = 9.0640(16)$ Å, $y = 0.199(2)$ and $z = 0.124(2)$ for phase I; $a = 3.0370(7)$ Å, $b = 2.8903(5)$ Å and $c = 5.2559(18)$ Å for phase II.

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Gigantic jets between a thundercloud and the ionosphere

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Transient luminous events in the atmosphere, such as lightning-induced sprites^{1–8} and upwardly discharging blue jets^{9–14}, were discovered recently in the region between thunderclouds and the ionosphere. In the conventional picture, the main components of Earth's global electric circuit^{15,16} include thunderstorms, the conducting ionosphere, the downward fair-weather currents and the conducting Earth. Thunderstorms serve as one of the generators that drive current upward from cloud tops to the ionosphere, where the electric potential is hundreds of kilovolts higher than Earth's surface. It has not been clear, however,

whether all the important components of the global circuit have even been identified. Here we report observations of five gigantic jets that establish a direct link between a thundercloud (altitude ~16 km) and the ionosphere at 90 km elevation. Extremely-low-frequency radio waves in four events were detected, while no cloud-to-ground lightning was observed to trigger these events. Our result indicates that the extremely-low-frequency waves were generated by negative cloud-to-ionosphere discharges, which would reduce the electrical potential between ionosphere and ground. Therefore, the conventional picture of the global electric circuit needs to be modified to include the contributions of gigantic jets and possibly sprites^{17,18}.

On the evening of 22 July 2002, an oceanic thunderstorm system was seen raging over the South China Sea near Luzon Island, Philippines (Fig. 1). Low-light-level cameras were set up at Kenting, which is located at the southern tip of Taiwan, to observe this thunderstorm. Between 14:09 and 14:21 UT, five gigantic optical jets were recorded above the thundercloud. At the fully developed stage, events 1 (Fig. 2) and 5 had a tree-shaped upper part and a blue-jet-like lower section, and hence are called 'tree' jets. For events 2, 3, 4 (Fig. 3), the upper section looked like a carrot, the lower section resembled a blue jet, and these are called 'carrot' jets.

Using the star field as a guide, the lines of sight of these events were found to intercept the convective core of the thundercloud. The obstructive low clouds (Figs 2 and 3) indicated that the gigantic jets emerged from the convective core, not from the front edge of the thundercloud. At this range, the corresponding heights of these jets were 90 ± 5 km (event 1), 89 ± 5 km (event 2), 86 ± 5 km (event 3), 91 ± 5 km (event 4) and 91 ± 5 km (event 5). All events reached

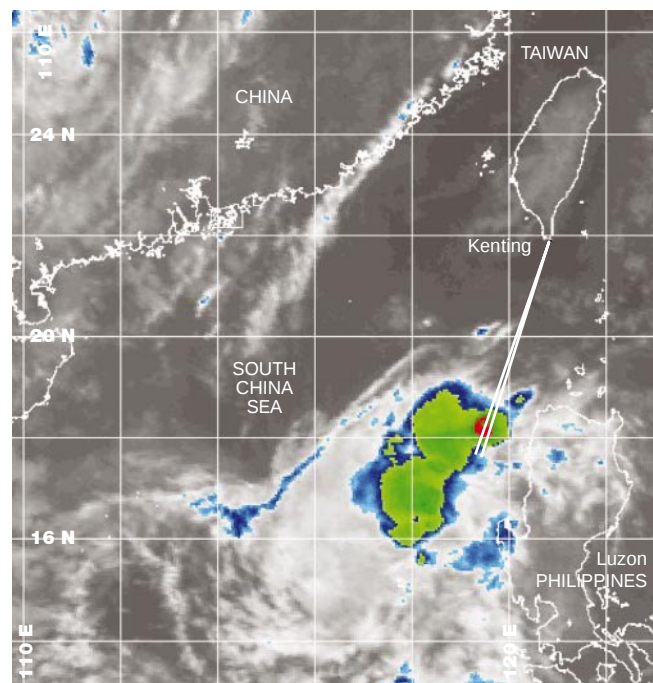


Figure 1 Infrared cloud map near Taiwan taken by the GMS 5 satellite at 14:31 UT, 22 July 2002. The occurrence times of five gigantic jets were 14:09:18 UT (event 1), 14:11:59 UT (event 2), 14:15:15 UT (event 3), 14:20:01 UT (event 4) and 14:20:54 UT (event 5). The two white lines represent the range of the line-of-sight extending from the observation site (at Kenting, Taiwan) to the centre of a gigantic jet. The convective core of the thunderstorm (red colour) is at a distance of 440 ± 20 km from Kenting. The colours on the infrared map represent the temperature and height of clouds, with red indicating the coldest and highest region. The cloud top inferred from the temperature profile is 16 km for the convective core.

the ionosphere, which has a night-time ionization starting at 80 km height and reaching the first peak density at 110 km, based on the International Reference Ionosphere (IRI-95) model¹⁹.

From the recorded images, the apparent emerging point of the jets was at 18 km height for event 2, which is only 2 km higher than the inferred cloud-top (16 km). For the jets shown in Figs 2 and 3, the apparent emerging points were 22 km and 24 km. The variation in emerging height was due to movement of the front cloud gap. Thus, these gigantic jets originated at the top of the convective core, and reached an elevation of 90 km in the ionosphere.

The images in Figs 2 and 3 are selected to illustrate the dynamical behaviours of the gigantic jets. The luminous duration was ~417 ms for event 1 (Fig. 2) and ~650 ms for event 4 (Fig. 3). The evolution of gigantic jets consists of three stages: leading jet, fully developed jet, and trailing jet. The propagating speed of the leading jets was ~1,000 km s⁻¹ for event 1 and ~1,200 km s⁻¹ for event 4. Both leading jets lasted only for two fields (each field is 17 ms). The form and evolution of the leading jets are similar to the blue jet recorded by Pasko *et al.*¹⁴, but had a much shorter duration. The propagation velocities of the leading jets reported here are an order of magnitude higher than those of classical blue jets and Pasko's jet, but are comparable to the velocities of stepped leaders in conventional cloud-to-ground lightning (CG)^{20,21}.

The fully developed jets lasted for less than 17 ms (Fig. 2) and for 167 ms (Fig. 3). At this stage, each gigantic jet appears to be a hybrid of sprite and blue jet, with its upper section, lower section, and apparent emerging point brightening concurrently. This feature is

similar to the return stroke in a CG^{20,21}, and the radio data, to be presented later, seem to support this assessment.

In Fig. 2, the trailing jet (field 5 and onward) was seen to rush upward with a speed of 26 km s⁻¹, and terminated at an elevation of ~60 km. The trailing jet lasted for 233 ms. After the trailing jet disappeared, the apparent emerging point re-brightened 17 ms later and lasted for two fields. The trailing jet in Fig. 3 propagated upward with a velocity of 120 km s⁻¹. As the trailing jet approached 60 km in elevation, its speed slowed down to 13 km s⁻¹ and it finally stopped at ~68 km. The persisting time of the trailing jet was 367 ms. The apparent emerging point also exhibited a re-brightening 167 ms later, and lasted for only one field. The trailing jets in all five events assume a conic shape with a ~25° conic angle, similar to the turbulence jet emerging from a nozzle²².

During this observation, synchronization of the recorder clock to global positioning system (GPS) time was done manually, and the time accuracy of the events was about a second. The uncertainty in time accuracy will not affect the results presented so far. But it did impose some difficulties when trying to correlate these optical events with CGs and extremely-low-frequency (ELF) transients.

From the ELF transients recorded by Onagawa station in Japan (Fig. 4) and Syowa station in Antarctica, a one-to-one match of the ELF event²³ to the jet was found for events 1, 2, 4 and 5. The ELF waves in all four cases have a polarization as generated by positive

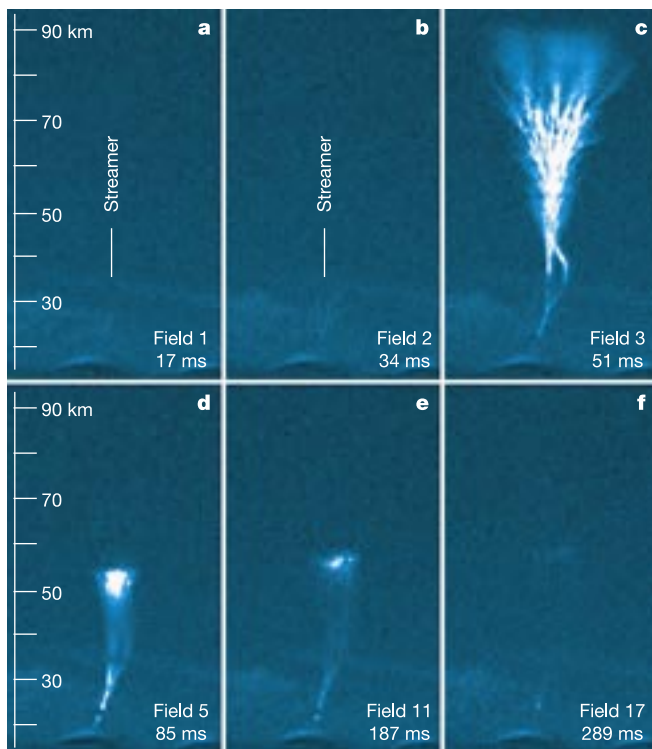


Figure 2 Selected and cropped image fields of event 1. The monochrome images were tinted to various shades of blue to bring out the salient structural features. The imaging system consists of a Watec N-100 CCD, a 20 mm/f1.8 lens, and a digital video recorder. Frame rate of the imaging system is 30 frames per second. Each frame was further separated into even and odd image fields of ~17-ms resolution. The spectral sensitivity of the CCD is from 400 to 1,000 nm, with 50% detection efficiency at 400 nm and at 780 nm. The persistent time of the Watec CCD's phosphor is less than 0.01 ms. (The cameras we used are intensified low light level cameras, which used a phosphor coating as image intensifier.)

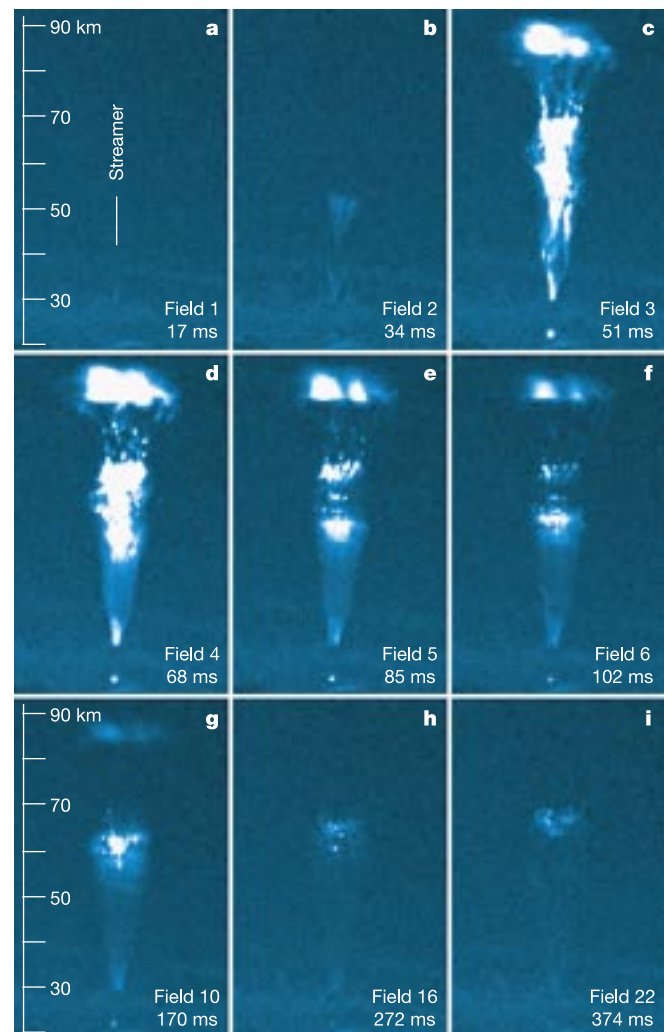


Figure 3 Selected and cropped image fields of event 4. The gigantic jet evolves in three stages: the leading jet (a, b), the fully developed jet (c-g) and the trailing jet (e-i).

CG (+CG) lightning. To locate the causative ELF sources, we performed triangulations¹⁷ using the data from the two ELF stations. The uncertainty in the ELF geolocating method is approximately 1,500 km. The results put one of the ELF causative sources in the general area of the jet-producing thunderstorm. However, the ELF transient time lagged behind the time of the optical event at the fully developed stage by 1.03, 1.01, 1.07 and 1.06 seconds. We believe these consistent time lags indicate that the recorder clock led the GPS clock of the ELF stations by about a second. Therefore, these four tree and carrot jets have their associated ELF transients. The charge moment changes¹⁸ inferred from ELF data were 1,700 and 2,000 C km for the two tree jets (events 1 and 5), and 1,000 C km for the two carrot jets (events 2 and 4). The difference in the charge moment change is consistent with the morphological difference of tree and carrot jets. A possible ELF transient for event 3 was found with a time lag of 0.59 seconds. Furthermore, the ELF geolocation puts the causative sources near Africa or North America. Therefore, no ELF was associated with this event.

Knowing the corrected times of optical jets, we can look for possible triggering CGs. We found no discernible lightning flash that preceded these gigantic jets. Next we examined data recorded by the TOGA lightning detection network²⁴, which operates in the western Pacific Rim. The TOGA network has a 10,000-km detection range and a 2-km positioning accuracy. We found no CGs that were close in time and in position to the jets. However, CGs could elude the detection of TOGA network when their current rise time was longer than 22 μ s. This feature is similar to classic blue-jet events^{11–13}, and it implies that CGs did not initiate these gigantic jets as they did for red sprites. Therefore, the associated ELF transients were probably generated at the fully developed stage of gigantic jets. At this stage, an electric path was set up between thundercloud and ionosphere, and current flowed through this conducting channel from the ionosphere toward the thundercloud. In this scheme, the fully developed stage behaved very similarly to the return stroke of CG lightning^{20,21}. A negative cloud-to-ionosphere (–CI) discharge would generate ELF waves with the +CG polarization observed in Japan and Antarctica. However, owing to the uncertainty in the recorder clock and possible misses of the TOGA system, there is a slight probability that some of the gigantic jets could be triggered by

+CG or a +CG occurred after the jets, and the observed ELF waves were produced by +CG and the jets.

Transient luminous events such as sprites and blue jets in the upper atmosphere have been studied extensively in the past decade^{1–14}. However, so far only six gigantic optical jets have been observed, and all were from oceanic thunderstorms. It is likely that the gigantic jets are a special feature of oceanic thunderstorms.

In a realistic model of global electric circuit¹⁶, the upper branch should be the whole atmosphere, which carries $\sim 2 \times 10^5$ C of charges. Most of the positive charges are confined to several tens of kilometres near the ground, and most of the $\sim 3 \times 10^5$ volts potential drop between the ionosphere and the ground^{15,16,20,21} also occurs in the same elevation. The traditional role of thunderstorms is to charge and to maintain the potential difference. In contrast, the gigantic jets, and possibly sprites^{17,18}, play a role in the global circuit by discharging the ionosphere and reducing the potential difference, with each jet removing ~ 30 C from the ionosphere. The charges removed by each jet account for $\sim 0.02\%$ of the total charged in the atmosphere but account for a substantial fraction of charges residing in the lower ionosphere. □

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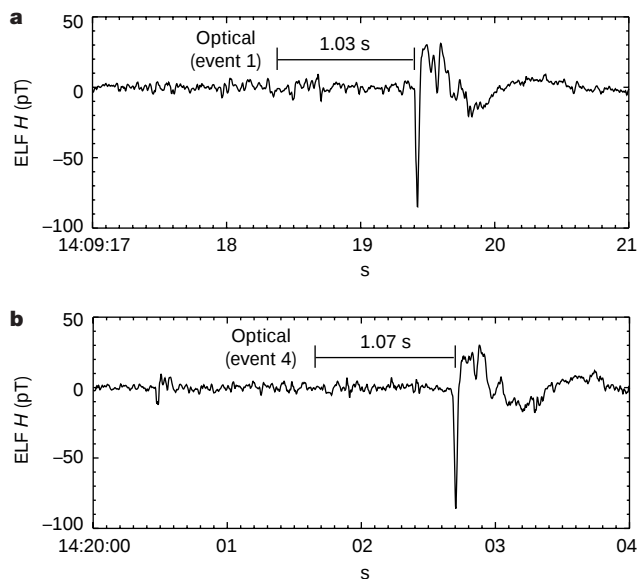


Figure 4 The *H*-component of ELF waves associated with the jets shown in Figs 2 and 3. The ELF transients were recorded at Onagowa (38.4° N, 141.5° E), Japan, on 22 July 2002.

Uranium series dates from Qesem Cave, Israel, and the end of the Lower Palaeolithic

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Israel is part of a geographical 'out of Africa' corridor for human dispersals. An important event in these dispersals was the possible arrival of anatomically modern humans in the Levant during the late Middle Pleistocene^{1–3}. In the Levant the Lower Palaeolithic ends with the Acheulo-Yabrudian complex, characterized by technological developments^{4,5}, including the introduction of technological innovations such as the systematic production of blades and the disappearance of hand-axes. These reflect new human perceptions and capabilities in lithic technology and tool function⁶. Qesem Cave, discovered in 2000, has a rich, well-preserved Acheulo-Yabrudian deposit holding great promise for providing new insights into the period. Here we report the dates of this deposit obtained by uranium isotopic series on associated speleothems and their implications. The results shed light on the temporal range of the Acheulo-Yabrudian and the end of the Lower Palaeolithic, suggesting a long cultural phase between the Lower Palaeolithic Acheulian and the Middle Palaeolithic Mousterian phases, starting before 382 kyr ago and ending at about 200 kyr ago.

Qesem Cave is situated 12 km east of Tel-Aviv, 90 m above sea level (32° 11' latitude, 34° 98' longitude). The cave's ceiling has been removed by natural erosion and recent construction work. Some of the cave deposits were damaged, but enough were preserved to justify a long-term field project. The cave was formed in Turonian limestone in the western foothills of the backbone mountain ridge of Israel under phreatic conditions⁷, and after later regional uplift it was dewatered and truncated by subaerial erosion. It has undergone several stages of natural and human-induced deposition, as well as subsidence and collapse. Natural deposits include calcite speleothems, bedrock collapse debris and clay fill, possibly originating from the overlying terra rossa soil. Speleothem deposition apparently occurred in this cave only before the last glacial cycle, probably because of a shift in the flow and dissolution processes of epikarstic water that followed the destruction of the roof. However, active speleothem deposition does occur in the region today, as it did during the last glacial and interglacial periods^{8,9}.

The 2001 salvage excavation exposed a stratigraphic sequence about 7.5 m deep that contained distinct archaeological horizons. Each of these yielded lithic assemblages in fresh condition and abundant faunal remains, all attributed to the Acheulo-Yabrudian complex of the terminal Lower Palaeolithic^{1,4,5}. The archaeological horizons showed differences in lithic technology and typology. Some are dominated by blades and blade-tools (Fig. 1a), whereas in others blades are rare or absent. Thick side-scrapers, the 'fossil directeurs' of Yabrudian industries (Fig. 1b), appear throughout the stratigraphic sequence. Hand-axes, the characteristic Acheulian tool type (Fig. 1c), appear in small numbers both at the top and at the bottom of the sequence, but not in every assemblage. The different lithic assemblages were found interspersed within the stratigraphic sequence, much like those in layer E at Tabun Cave and Yabrud I^{10–13}. At the base of the cave, above bedrock, typical Acheulo-Yabrudian tool types are rare. Chopping tools and spheroids, typical of Acheulian industries, were found in these assemblages. It

seems that the earliest occupation at Qesem predates the Acheulo-Yabrudian.

The chronology of the upper layers of Qesem Cave is based on speleothems from the eastern section of the cave. These were sampled with a cutting disk and their ²³⁰Th–²³⁴U dates were measured by thermal ionization mass spectrometry (TIMS) at the uranium-series laboratory of Bergen University (Table 1). After conventional chemical preparation, mass abundances of natural U and Th isotopes were measured against a mixed ²³⁶U–²³³U–²²⁹Th spike¹⁴. Ages were calculated with the aid of the TIMS–Age 4U2U program¹⁵ and corrected for thorium detrital content, assuming an initial ²³⁰Th/²³²Th ratio of 1.5 (ref. 16). Field relations indicate that the ages were in correct stratigraphic order (Fig. 2). We identified two main stages of speleothem deposition. The first, a massive (about 25 cm thick) flowstone deposit, is dated by five ages (in kyr): 382 ± 37, 300 ± 13, 218 ± 15, 218 ± 16 and 207 ± 12. This flowstone covers the lower Acheulo-Yabrudian layers. A detached part of the flowstone, dated to 254 ± 37 kyr ago, was redeposited in an archaeological breccia deposit, indicating that the breccia is younger. A break in speleothem deposition occurred between about 207 and 152 kyr ago. Within this period the latest human occupation of the cave might have taken place, indicated by thin archaeological sediment directly above the massive flowstone. A second, short period of speleothem deposition took place about 152 kyr ago, represented by two ages of a calcite crust a few millimetres thick, and small stalactites, dated to 152 ± 3 and 152 ± 7 kyr ago. The crust and the stalactites were deposited over the archaeological sediments and can therefore serve as a terminus of human occupation. The major Acheulo-Yabrudian occupations

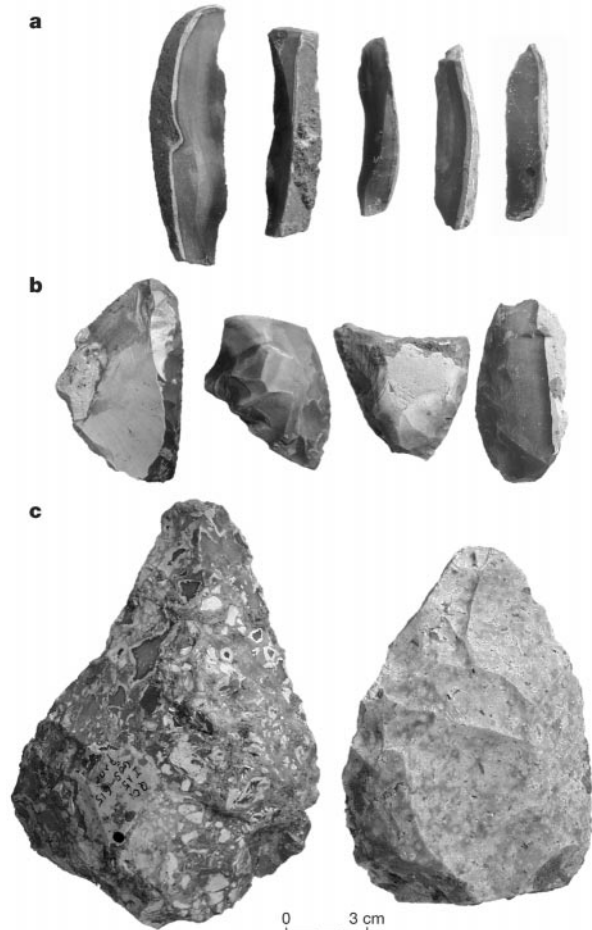


Figure 1 Flint tools from Qesem Cave. a, Backed blades; b, side-scrapers; c, hand-axes.

Table 1 Isotopic data and ages of speleothems in Qesem Cave

Sample	^{238}U (p.p.m.)	$^{230}\text{Th}/^{234}\text{U}$	$^{234}\text{U}/^{238}\text{U}$	$^{230}\text{Th}/^{232}\text{Th}$	Initial $^{234}\text{U}/^{238}\text{U}$	Age (kyr)	Corrected age (kyr)
507 (Q5)	0.36	0.7711 ± 0.0146	1.0266 ± 0.0074	19.51 ± 0.58	1.0417 ± 0.0075	$158.52^{+7.50}_{-6.96}$	$151.97^{+7.55}_{-7.02}$
501 (Q11-1)	0.78	0.8864 ± 0.0116	1.0869 ± 0.0324	61.96 ± 1.28	1.1624 ± 0.0346	$220.73^{+15.11}_{-12.53}$	$218.52^{+15.11}_{-12.53}$
502 (Q10-1)	0.29	0.9284 ± 0.0212	1.0829 ± 0.0300	23.58 ± 0.37	1.1732 ± 0.0325	$259.98^{+37.06}_{-26.34}$	$253.88^{+37.07}_{-26.34}$
503 (Q3B1)	1.74	0.8698 ± 0.0130	1.0907 ± 0.0183	149.90 ± 1.31	1.1636 ± 0.0195	$208.10^{+12.02}_{-10.56}$	$207.21^{+12.02}_{-10.56}$
504 (Q2)	0.33	0.7624 ± 0.0054	1.0409 ± 0.0143	94.51 ± 0.75	1.0633 ± 0.0146	$153.73^{+3.29}_{-3.12}$	$152.43^{+3.29}_{-3.12}$
528 (Q10-2)	0.18	0.8890 ± 0.0171	1.0543 ± 0.0047	13.98 ± 0.36	1.1037 ± 0.0049	$228.06^{+16.53}_{-14.30}$	$217.65^{+16.61}_{-14.38}$
526 (Q3D1)	0.24	0.9534 ± 0.0065	1.0470 ± 0.0034	24.45 ± 0.38	1.1120 ± 0.0036	$305.87^{+13.53}_{-11.97}$	$299.70^{+13.58}_{-12.03}$
569 Q3D2	0.18	0.9808 ± 0.0057	1.0299 ± 0.0092	78.00 ± 1.10	1.0888 ± 0.0097	$383.94^{+36.73}_{-26.65}$	$381.98^{+36.73}_{-26.65}$

TIMS ages were obtained with a Finnigan 262 RPQ mass spectrometer. Laboratory errors are reported with two standard deviations; $^{230}\text{Th}/^{232}\text{Th}$ ratios of more than 20 indicate that radiogenic ^{230}Th predominated and detrital contamination was not significant. Initial $^{234}\text{U}/^{238}\text{U}$ is stable over time, at 1.044 ± 0.038 (2σ), indicating that the calcite behaved as a closed system. Ages were corrected on the assumption that the initial $^{230}\text{Th}/^{232}\text{Th}$ ratio was 1.5.

of the cave therefore began well before about 382 kyr ago, probably during oxygen isotope stage 11. It ended before 152 kyr ago, possibly shortly after 207 kyr ago. Between about 382 and 207 kyr ago, human occupation could have taken place simultaneously with speleothem deposition, or during drier intermediate periods.

The Acheulo-Yabrudian complex contains three major lithic facies defined at Yabrud I in Syria^{5,13} and at Tabun Cave in Israel^{10,11} in the 1930s and 1950s. These were termed as follows: Yabrudian is dominated by thick scrapers shaped by steep Quina retouch; Acheulo-Yabrudian contains Yabrudian scrapers and handaxes; and Pre-Aurignacian/Amudian is dominated by blades and blade-tools^{5,13}. A renewed excavation at Tabun viewed these different facies as representing a gradual technological change within what was suggested as ‘the Mugharan tradition’¹². In 1925 the cave of Zuttiyeh in Wadi Amud (Israel) was excavated and part of a human skull (‘Galilee Man’) was found¹⁷. A renewed excavation at Zuttiyeh confirmed an Acheulo-Yabrudian occupation at the site¹⁸. This unique human fossil was seen as representative of the makers of Pre-Mousterian assemblages^{19,20}. Recently it was suggested that the Zuttiyeh skull should be recognized as an anatomically modern human²¹, thus suggesting a possible link between physical evolution and lithic technology. A few Acheulo-Yabrudian sites are known from Lebanon, Syria⁵ and Israel (recently reported from Jamal cave²² and Mysliya in Mount Carmel). The discovery of Qesem Cave, being the southernmost Acheulo-Yabrudian site yet found, extends the known geographic boundaries of this complex.

The Acheulo-Yabrudian was originally recognized by its characteristic lithic assemblages and its place in the Levantine prehistoric

sequence. When in stratigraphic context, as at Tabun Cave, it is above late Lower Palaeolithic (Upper Acheulian) and below early Middle Palaeolithic (Mousterian) layers¹⁰. Absolute chronology for the Acheulo-Yabrudian has been difficult because almost all excavations predate the advent of modern radiometric techniques. However, in recent years archaeologists have provided series of radiometric dates of both Lower and Middle Palaeolithic sites in the Levant and made some attempts to date Acheulo-Yabrudian layers^{22–28} by uranium isotopic series, thermoluminescence (TL) and electron spin resonance (ESR).

The oldest dates available for the Acheulo-Yabrudian complex are about 350 kyr and the most recent are up to 160 kyr. Early Middle Palaeolithic Mousterian deposits produced a few dates as early as about 200 kyr at Tabun and Hayonim caves and Rosh Ein Mor^{23,29}. Although single dates for final Acheulo-Yabrudian seem to partly overlap the early Mousterian, we do not believe that this was so. A date of 400 kyr from Tabun layer E was recently obtained (W. J. Rink, H. P. Schwarcz, A. Ronen and A. Tsatskin, personal communication) and the Acheulo-Yabrudian might therefore have started before 350 kyr ago and ended about 200 kyr ago (Fig. 3).

Mainly on the basis of ESR dates from several sites, it was recently suggested that the Lower–Middle Palaeolithic transition was rapid, taking place at 215 ± 30 kyr ago²⁷. Because these dates were

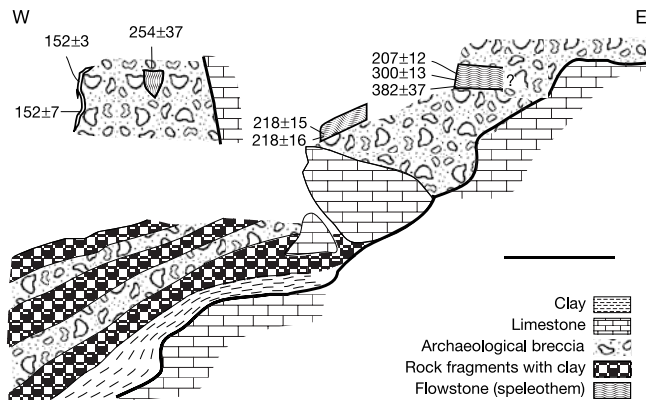


Figure 2 Schematic compiled section showing field relations and dates at the eastern side of Qesem Cave. The inset shows an additional section at the southeastern part of the cave, beyond the plane of the main section. Both parts are shown at the same relative elevation. Limestone blocks are associated with bedrock collapse and subsidence. Approximate uranium-series dates and errors (in kyr) of speleothems indicate the upper and lower limits of the age of the upper archaeological layers. For precise dates see Table 1. Scale bar, 1 m.

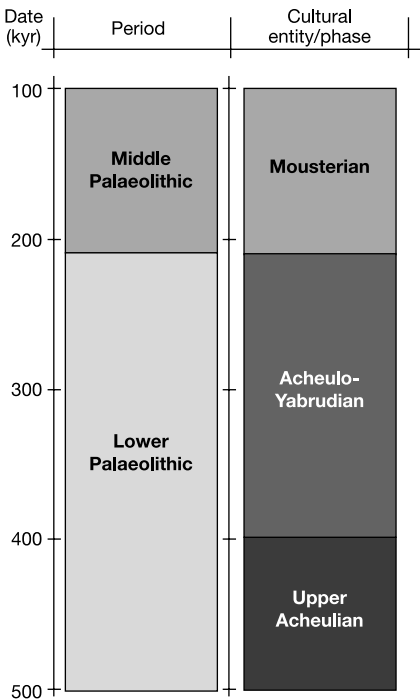


Figure 3 Palaeolithic chronological phases and cultural units in the Levant.

measured long after the excavations, however, appropriate on-site dosimetry was not available and it was suggested that these ESR dates underestimate the age of the teeth²⁴. Although a date of about 200 kyr is acceptable for the end of the Acheulo-Yabrudian, the dates from Tabun and Qesem caves indicate a very long and dynamic cultural complex covering about 200 kyr between the two major Palaeolithic complexes, the Lower Palaeolithic Acheulian and the Middle Palaeolithic Mousterian.

Our study is the first to date Acheulo-Yabrudian deposits by the method of ²³⁰Th/²³⁴U TIMS. On the basis of samples extracted during the archaeological excavation, an exact stratigraphic association of the dates can be made. Three main points emerge from the findings. First, the Acheulo-Yabrudian complex probably started well before 382 kyr ago, presumably during oxygen isotope stage 11. Because we have not yet dated the lower parts of the sediments in Qesem Cave, it would be fair to assume dates that accord well with the early Tabun E dates up to 400 kyr. Second, Acheulo-Yabrudian sediments at Qesem Cave are covered by a speleothem dated to 152 kyr. The Acheulo-Yabrudian occupation in Qesem Cave therefore ceased long before that date. Third, the dates of Qesem Cave represent the last cultural phase of the Lower Palaeolithic, the Acheulo-Yabrudian complex, supporting a maximum age limit of about 207 kyr ago for the earliest stages of the Middle Palaeolithic Mousterian complex. No traces of Mousterian occupation were found at Qesem.

The rich Acheulo-Yabrudian deposits at Qesem Cave offer a rare opportunity to study human adaptation and evolution in the Middle Pleistocene. Because the dates indicate that human activity occurred mostly before 382 kyr, and because the site is located within the 'out-of-Africa' corridor, the information obtained by a study of Qesem Cave is likely to contribute substantially to our understanding of the origins and dispersal of modern humans². The Levantine Acheulian assemblages predating the Acheulo-Yabrudian were probably made by *Homo erectus* (*sensu lato*), whereas Mousterian industries postdating the Acheulo-Yabrudian were made by both anatomically modern humans and *Homo neanderthalensis*. It would be interesting to learn who was the maker of the unique Acheulo-Yabrudian assemblages³. If human remains are recovered, Qesem might hold a key to the understanding of evolution and dispersal of modern humans. The stratigraphically distinct archaeological horizons at Qesem have already provided, and will continue to provide, information on the late Lower Palaeolithic Acheulo-Yabrudian lithic variability. Such knowledge will improve our understanding of technological innovations, such as the beginning of the systematic production of blades in the Levant^{4,6} and the early stages of the Levallois technique, a technological breakthrough that became globally prominent in the Middle Palaeolithic. In contrast, this period also saw the final stages of hand-axe manufacture, a tradition that had accompanied humans for about one and a half million years. □

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The evolution of reproductive isolation through sexual conflict

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Classical population-genetics theory suggests that reproductive isolation will evolve fastest in small isolated populations¹. In contrast, recent theory suggests that divergence should occur fastest in larger allopatric populations². The rationale behind this is that sexual conflict, potentially the strongest driver of speciation, is greater in larger, higher-density populations. This idea is highly controversial³ and has little experimental support^{4,5}. Here we show, using replicate fly populations with varying levels of sexual conflict, that larger, more dense populations with more sexual conflict diverged to a greater degree than small populations with relaxed conflict. This result strongly suggests that speciation can occur rapidly in large populations through increased sexual conflict.

Sexual conflict is a potent evolutionary force that may lead to

cycles of sexually antagonistic coevolution, with adaptations in one sex provoking counter-adaptations in the other^{6,7}. These antagonistic cycles are implicated in the rapid evolution of some reproductive proteins, the fastest-evolving proteins known^{7,8}. Sexually antagonistic coevolution is particularly interesting for its potential to drive fast evolutionary change and cause allopatric population divergence⁹. This occurs because reproductive genes evolve under sexual conflict, and hence during periods of allopatric divergence, reproductive incompatibilities should swiftly accumulate. This mechanism is considered to be a widely applicable sexual-selection speciation engine¹⁰.

To investigate population divergence resulting from sexual conflict, we used experimental evolution with replicate populations of the dung fly *Sepsis cynipsea*. We used three treatments: high density, low density and monogamy. In high-density and low-density populations, flies were kept in large containers and could interact freely so that both pre- and postcopulatory sexual selection, including sexual conflict, were possible. Increasing numbers of compatible males in larger populations caused increased conflict in theoretical investigations². Here it is the number of sexual interactions *per se*, but nonetheless, in the model² and here, conflict was elevated at higher population density. Monogamous populations consisted of pairs with lifelong monogamy and random pairing within lines, and represent populations where sexual conflict was absent. After 35 generations of evolution under different levels of conflict followed by two generations of relaxed selection, population divergence was assessed based on mating behaviour. Flies were paired either within or between populations but always within treatments. Behaviour was analysed with the proportion of copulations or reluctance behaviour as dependent variables, and selection (monogamous, low density or high density) and mating type (within or between (populations of the same treatment)) as predictors. As shown in Fig. 1, there were highly significant effects of both predictors and their interaction on the proportion of copulations (selection: $F_{2,12} = 147.28$, $P = 0.0001$; mating type: $F_{1,12} = 63.54$, $P = 0.0001$; selection $\times$ mating type: $F_{2,12} = 27.55$, $P = 0.0001$). Higher proportions of successful copulations were found in focal pairs from monogamous populations compared with those from the conflict treatments (low-density and high-density populations). Additionally, there was no difference between the mating proportions in pairs formed from within or between allopatric monogamous populations (Fig. 1). In contrast, there were pronounced differences in the conflict treatments. Copulation frequency was greater in pairings within conflict populations than those between populations, and this effect was stronger in

high-density than in low-density populations (Fig. 1). Analysis of female resistance behaviour (proportion of females shaking to dislodge males) confirms this (Fig. 2). More successful cross types (those with more copulations) are also those where female resistance occurs more rarely (selection: $F_{2,12} = 137.38$, $P = 0.0001$; mating type: $F_{1,12} = 127.97$, $P = 0.0001$; selection $\times$ mating type: $F_{2,12} = 37.60$, $P = 0.0001$; Fig. 2). Notably, this demonstrates that low copulation levels are not due to lack of male attempts, but rather to rejection by females. These results indicate that copulation frequency decreased and female resistance behaviour increased with increasing sexual conflict. Furthermore, in pairings between different populations (within conflict treatments), females showed greater resistance and copulated less than within populations, indicating female preference for males from their own population. Why females are more reluctant to mate with males from other conflict populations is uncertain. Presumably there is fitness variation underlying this behaviour, although this remains to be explored.

Consistent with theory^{2,11} there was no indication of increased reproductive isolation between allopatric monogamous populations. Divergence was not expected to be as pronounced in the absence of conflict (that is, under monogamy). Finally, the effects on copulation and female resistance frequency were more pronounced in crosses between different high-density populations than in those between low-density populations (Post-hoc testing of the main models using Fisher's partial least significant difference (PLSD); high density (between) versus low density (between): copulation, $P = 0.0001$; shaking, $P = 0.0006$; Figs 1 and 2). In both low-density and high-density populations, frequencies of copulation were significantly lower and female resistance more pronounced in crosses between populations than within populations. However, when females were crossed within their own populations, high-density and low-density populations did not differ significantly from each other (Fisher's PLSD; high density (within) versus low density (within): copulation, $P = 0.39$; shaking, $P = 0.07$). These results indicate that increased behavioural (precopulatory) reproductive isolation has evolved between high-density compared with between low-density or monogamous populations, and indicates that population density had a role in driving reproductive isolation by intensifying sexual conflict². Unlike in the model², however, divergence in our populations must have occurred using standing genetic variation rather than new mutations.

Comparative evidence also indicates that conflict leads to increased speciosity¹², although this has been contested¹³. Nevertheless, the experimental results presented here demonstrate the

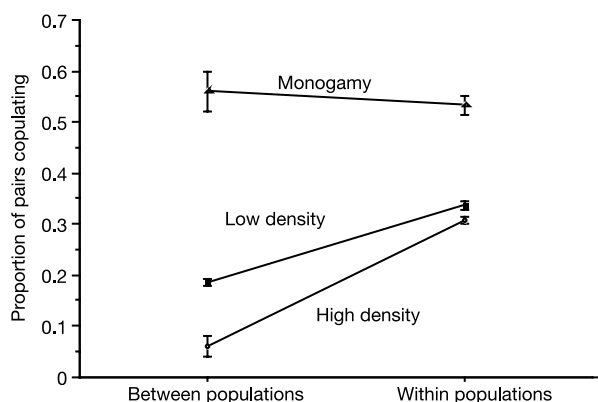


Figure 1 Interaction plot showing the effects of treatment (monogamy, low-density or high-density populations) and mating type (within populations or between populations of the same treatment) on the proportion of pairs that copulated. Error bars indicate ± 1 standard error.

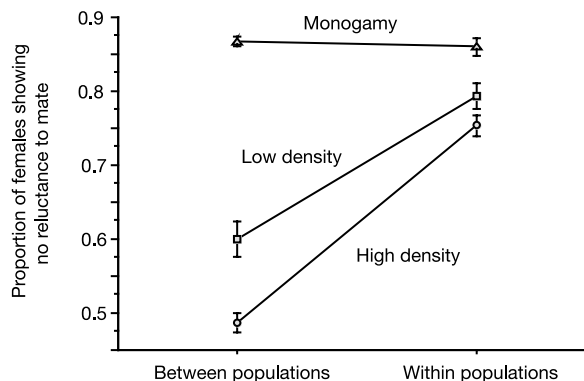


Figure 2 Interaction plot showing the effects of treatment (monogamy, low-density or high-density populations) and mating type (within populations or between populations of the same treatment) on female reluctance (reluctance to mate is stereotypical and violent shaking behaviour). The proportion of females showing no reluctance to mate is displayed to facilitate comparison with Fig. 1. Error bars indicate ± 1 standard error.

importance of sexual conflict as a potential driver of speciation. Our results also contradict classical views of speciation¹, as divergence was faster in large (rather than small) populations. This result seems especially sound because potential inbreeding differences between populations should lead to lower mating rates in monogamous or low-density populations^{14–16}. Notably, even if inbreeding had any influence, this would only alter conclusions concerning the effect of treatment on copulation frequency. The differences between matings within and between populations and the greater precopulatory reproductive isolation between high- than between low-density populations are unaffected. These cannot be explained by differential inbreeding alone, and the main conclusions would therefore remain unchanged. This seems to be especially true because low-density and monogamous populations have very similar population sizes and yet they differ markedly. Evidence of female (but not male) resistance to flies from other conflict populations implies that sexual conflict drove divergence. This asymmetry in responses is unlikely to be driven by differential natural selection. Additionally, divergent natural selection is not expected to cause populations under identical selection necessarily to diverge¹⁷, but the pattern we see here is an explicit prediction of divergence through sexual conflict². Nevertheless, other forms of sexual selection (for example, female choice) could potentially generate these results¹⁸. However, considering what is known of *S. cynipsea*^{19–22}, the pattern detected seems best explained by sexual conflict.

We have shown that increased population density/size increases the frequency of sexual interactions and thus sexual conflict. This increased sexual conflict caused a rapid increase in behavioural reproductive isolation, especially in larger, high-density populations, confirming the importance of conflict as a driving force in evolution. This provides experimental evidence for this new concept², which contrasts with classical population-genetics theory. □

Methods

Mating behaviour

Sepsis cynipsea is ideally suited for testing models of speciation by means of antagonistic processes because sexual conflict is blatantly obvious, with long and violent precopulatory struggles^{19–22}. Conflict over copulation is due to injuries that males inflict on females during copulation, which reduce female fitness²². Males appear to largely control copulation duration²³, and longer copulations further reduce female fitness²⁴. Previous work has also shown that female mating rates increase with the number of males present²⁵, and that females are more likely to re-mate with different males²⁶. Additionally, copulation greatly increases female mortality²².

Density and frequency of sexual interactions

We ensured that increased population size/density indeed led to increased sexual activity, as required by theory. Assays of the effects of population density on the frequency of sexual interactions were performed with unselected flies. Virgin one-week-old males were placed together with females in containers (standard size) with differing population densities (one container each with either 1, 5, 10, 25, 50 or 100 individual(s) of each sex). Over a period of 8 h the population cages were checked every hour and the number of copulating pairs and females exhibiting reluctance behaviour (vigorous shaking) were counted. From this the frequency of copulations, female reluctance behaviour and total sexual interactions were calculated. We found that density/fly number significantly increased the frequency of copulations and sexual interactions (repeated measures analysis of variance (ANOVA; controlling for number of flies present) indicated significant effects of density on the number of copulations ($F_{5,20} = 7.18$, $P = 0.0005$) and sexual interactions ($F_{5,20} = 5.04$, $P = 0.004$). In total, five assays (including the six different densities in parallel) were performed. These findings indicate that at higher population densities, polyandry and hence sexual conflict increased. Additionally, housing females with males after a first copulation also lowers female fitness (lifetime offspring production: single copulated female housed alone = 78.8 ± 13.0 ; single copulated females housed with three males = 27.0 ± 6.4 ; analysis of co-variance (ANCOVA), $F_{1,37} = 11.4$, $P = 0.002$). This, in addition to previous data²⁷, shows that increasing the number of males that a female interacts with reduces her fitness, consistent with increased sexual conflict.

Selection lines

Stock populations were founded using male and female *S. cynipsea* collected at Fehrltorf in 2000 and maintained in population cages for five generations to allow acclimatization to laboratory conditions. Selection lines were started simultaneously including three treatment types each with three replicate populations. These consisted of high-density (HD1, 2 and 3, each with 250 males and 250 females per container), low-density (LD1,

2 and 3, each with 25 males and 25 females per container) and monogamous populations (M1, M2, M3, each consisting of 20 pairs in individual vials). High-density and low-density populations were maintained in large containers (same size for both treatments). For the monogamous populations, emerging offspring were sexed and randomly paired in 100-ml vials. The populations of all three treatments were given sugar, pollen and water *ad libitum* and supplied with fresh dung in small quantities every 3 days. However, the numbers of food, water and dung containers were varied so that they remained approximately proportional to the number of flies present. Importantly, by standardizing rearing conditions (food, temperature, relative humidity and photoperiod) across treatments, differential natural selection was not expected. Natural selection through density differences should be reflected in body size changes, as fly size responds rapidly to food or dung shortages²⁸. However, we found no significant differences in body size across our selection regimes (female size: $F_{2,6} = 0.92$, $P = 0.45$; male size: $F_{2,6} = 0.22$, $P = 0.80$).

Flies were left to interact, and after approximately 12 days (30–60% of average laboratory lifespan) larger portions of dung were provided. These dung portions were collected and pooled per population and emerging flies were separated by sex. Flies for the next generation were taken randomly from these pooled offspring, which were always produced in larger numbers than required for maintaining populations. This allowed differential reproduction and ensured that the most successful genotypes per treatment (those which produce more offspring) were represented to a greater degree in the subsequent generation. We also synchronized the starting times of each generation between treatments to avoid differential selection on developmental times. All flies were maintained in the same large climate chamber (23–27 °C, 60–70% relative humidity, 12-h light/dark photoperiod), and cage and vial placement changed regularly to avoid microclimate effects.

Experiments

We investigated the effects of sexual conflict on population divergence in replicate laboratory *S. cynipsea* populations. After 35 generations of selection (different levels of conflict), flies were housed under relaxed selection for two generations to eliminate differential maternal effects and potential phenotypic effects generated by population density variation (all populations from all treatments in containers with 50 males and 50 females and kept for 12 days per generation as in the selection populations). The offspring of these flies were used for the experiments. To investigate population divergence, experimental females were placed separately with single males from the same treatment but from their own (within) or different populations (between) for 30 min and mating behaviour was assessed by direct observation. The numbers of females to copulate (only copulations with normal duration and uninterrupted genital contact) or show reluctance behaviour (vigorous shaking) were counted. In subsequent analyses, cross type (monogamous × monogamous, low density × low density or high density × high density) was the unit of replication and consisted of pairings between populations or within populations. For each cross type the behaviour of 50 females was observed (for example, 50 M1 females with M1 males for pairings within the M1 population or 25 M1 females with 25 M2 males plus 25 M2 females with 25 M1 males for pairings between populations M1 and M2, and so on).

Data analysis

Data were analysed using a general linear model. Before analysis, proportion data were arcsin square-root-transformed. Residuals from all analyses did not differ significantly from a normal distribution (Kolmogorov–Smirnov tests, all $P > 0.05$).

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Opposing basal ganglia processes shape midbrain visuomotor activity bilaterally

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The manner in which the nervous system allocates limited motor resources when confronted with conflicting behavioural demands is a crucial issue in understanding how sensory information is transformed into adaptive motor responses. Understanding this selection process is of particular concern in current models of functions of the basal ganglia¹. Here we report that the basal ganglia use simultaneous enhancing and suppressing processes synergistically to modulate sensory activity in the superior colliculi, which are bilaterally paired midbrain structures involved in the control of visual orientation behaviours². These complementary processes presumably ensure accurate gaze shifts mediated by the superior colliculi despite the presence of potential distractors.

Each superior colliculus (SC) contains a map-like representation of contralateral visual space that is in register with a motor map that produces movements of the head and eyes (or gaze shifts) to targets within that region of visual space³. Thus, the right SC controls gaze shifts to the left visual space, whereas the left SC effects movements to the right visual space. The specific vector of a gaze shift depends on the topographical locus of neural activity that the visual target induces within the contralateral SC³. This 'retinal error' signal is conveyed to brainstem regions that ultimately produce a gaze shift

that brings the central retinae to bear on the target⁴. The basal ganglia are intimately involved in these SC-mediated processes through output neurons in the substantia nigra, pars reticulata (SNr)^{5–8}. Two populations of nigrocollicular neurons are present anatomically^{9,10}, a robust uncrossed projection and a smaller crossed component.

Uncrossed neurons have high rates of spontaneous activity^{5–8}, and because they use the inhibitory neurotransmitter GABA (γ -aminobutyric acid)¹¹, activity in the SC output neurons that they contact^{12,13} is normally suppressed⁵. However, before a gaze shift to a target in, for example, the right visual space, there is a temporary cessation of activity in a subset of uncrossed nigrocollicular neurons in the left SNr^{7,8}. This phasically releases a corresponding subset of ipsilateral SC output neurons from inhibition and facilitates their activity; it is then conveyed to the contralateral brainstem and spinal cord^{7,8}. These regions, in turn, activate the motor nuclei innervating the extraocular and neck muscles necessary to shift gaze to the desired location in space. This process of 'disinhibition' functions not only as the basic template by which the basal ganglia influence SC-mediated visuomotor behaviours, but also as the mode by which they interact with other motor-related structures¹⁴. However, nothing is known about the physiology of the crossed nigrocollicular neurons. Here we report that these neurons have unique physiological properties, and we suggest a potential role for the basal ganglia in the interhemispheric coordination of SC activity related to visuomotor behaviours.

Single-unit extracellular recordings were made from visually responsive neurons ($n = 303$) in the left SNr of anaesthetized cats. Of these neurons, 59 were antidromically activated from one or the other SC and were selected for quantitative analysis. They fell into two populations: one antidromically activated from the left SC and thus defined as uncrossed ($n = 43$; 73%) and another ($n = 16$; 27%) antidromically activated from the right SC and defined as crossed. No SNr neurons were encountered that could be antidromically activated from both SCs, indicating that these are segregated populations.

The two neuronal populations had different spatial distributions. All crossed neurons were recorded in the anterolateral aspect of SNr, at stereotaxic loci (anterior to posterior, +5.2–6.3 mm) where lesions have been shown to ameliorate cortically induced visual hemineglect¹⁵. In contrast, uncrossed nigrocollicular neurons were distributed over a broad rostrocaudal extent of SNr, which is consistent with anatomical reports⁹. The two neuronal populations also differed in conduction velocities. Assuming an average conduction distance of 7 and 13 mm, respectively, the conduction velocities of the uncrossed neurons were significantly lower (5.38 ± 2.036 (s.d.) m s^{-1} ; range 1.98–10.4 m s^{-1} ; $P < 0.01$; two-tailed t -test) than those of crossed neurons (mean 8.98 ± 3.699 m s^{-1} ; range 3.64–14.2 m s^{-1}).

Despite these differences, all ($n = 59/59$; 100%) were orthodromically activated from regions of the ipsilateral lateral suprasylvian cortex, indicating that visual cortical activity can influence the SC by means of transynaptic corticofugal pathways that course through the basal ganglia. The patterns of activation observed in crossed and uncrossed neurons after orthodromic stimulation of extraprimary visual cortex were similar to those observed after electrical stimulation of frontal and motor cortex¹⁶.

Uncrossed neurons displayed high rates of spontaneous activity (36.81 ± 18.08 Hz; range 11.06–86.26 Hz), consistent with their tonic inhibition of ipsilateral SC neurons^{7,8}. Their visual receptive fields were relatively small (876 ± 443.11 deg²; range 340–5,225 deg²), with centres confined within the contralateral hemifield. There was also good spatial correspondence between their receptive fields and those of neurons at effective antidromic stimulation sites in the ipsilateral SC. Both had their receptive fields centred in contralateral space and in topographic register with each other ($n = 43/43$; 100%). All uncrossed neurons encountered

($n = 43/43$; 100%) were phasically inhibited by visual stimuli (Fig. 1).

By contrast, all crossed nigrocollicular neurons ($n = 16/16$; 100%) were phasically excited by visual stimuli (Fig. 2), indicating that the same visual stimulus has opposite effects on these two populations. Furthermore, the spontaneous activity of crossed neurons (12.52 ± 10.29 Hz; range 0.12–40 Hz) was lower than that of uncrossed neurons ($P \ll 0.001$; two-tailed t -test). In some crossed neurons, the absence of spontaneous activity required that their initial localization be based solely on antidromic activation. This is consistent with the absence of strong tonic crossed nigrocollicular influences. The visual receptive fields of crossed neurons ($7,499 \pm 3,068.46$ deg²; range 3,080–12,100 deg²) were significantly larger than those of their uncrossed counterparts ($P \ll 0.001$; two-tailed t -test). There was also a mismatch between the receptive fields of crossed neurons and the SC neurons at the effective antidromic stimulation sites. In all instances ($n = 16/16$; 100%), receptive field centres of crossed neurons were in the hemifield opposite those recorded at the effective SC antidromic activation site(s). Many ($n = 14/16$; 87%) crossed neurons were antidromically activated from all four SC stimulation sites, suggesting that, when activated, crossed neurons can exert their influence over larger regions of their target SC than can their uncrossed neighbours.

Although it is assumed that SNr output neurons use the inhibitory neurotransmitter GABA, excitatory neurotransmitters are known to be present in some SNr output systems¹⁷. To determine whether crossed neurons use the same neurotransmitter as their uncrossed counterparts¹¹, wheat-germ agglutinin–horseradish peroxidase (WGA–HRP) was injected into one SC and the tissue was processed simultaneously for glutamic acid decarboxylase (GAD) immunohistochemistry, an enzymatic marker for GABA-

mediated neurons. All crossed somas retrogradely labelled with HRP were also immunoreactive for the anti-GAD antibody (Fig. 3a, c), indicating that they, like their uncrossed counterparts, are GABA-mediated. However, there were far fewer crossed than uncrossed neurons, and counts of retrogradely labelled SNr neurons revealed an uncrossed-to-crossed neuron ratio of about 12:1 (ref. 9).

For crossed neurons to have a direct effect on SC-mediated behaviours, they should terminate on the descending SC output neurons that project to the brainstem and spinal cord. In the cat, these ‘colliculoreticulospinal’ neurons contain the calcium-binding protein parvalbumin¹⁸. To evaluate this possibility, we combined injections of the anterograde tracer biotinylated dextran amine in the rostral SNr with parvalbumin immunohistochemistry. Labelled fibres of crossed neurons coursed deep within the ipsilateral SC, following the contour of the periaqueductal grey to enter the opposite SC. Their distinct trajectory is consistent with our inability to activate crossed neurons with the low stimulus current (100–300 μ A) presented in the intermediate laminae of the ipsilateral SC (see above). In the intermediate and deep laminae of the contralateral SC, labelled boutons were observed in close apposition to the soma and proximal dendrites of parvalbumin immunopositive neurons (Fig. 3b, d), indicating that crossed neurons establish direct contact with contralateral colliculoreticulospinal neurons controlling shifts of gaze⁴.

The consequences of crossed neuronal activity on responses of their target neurons in the contralateral SC then were evaluated by recording sensory-evoked activity through the SC ‘stimulating’ electrodes while orthodromically stimulating the SNr through the ‘recording’ electrode. The recordings were made through the SC electrode yielding the lowest antidromic threshold for a given crossed nigrocollicular neuron while stimulating at the locus of

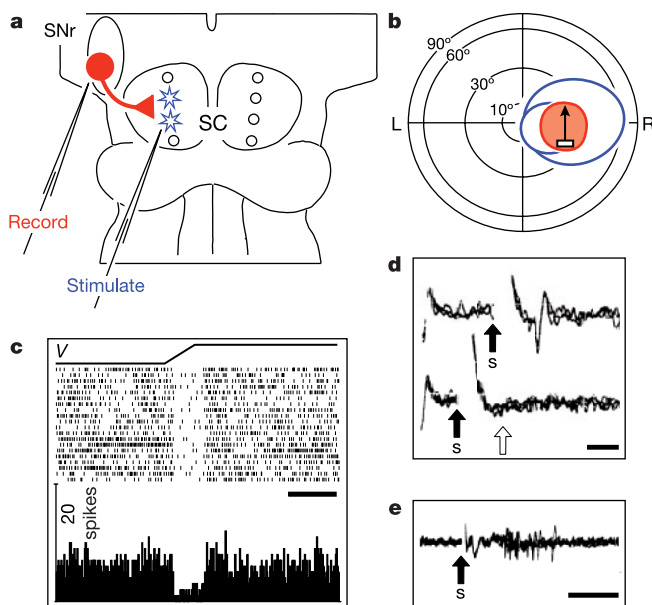


Figure 1 Uncrossed nigrocollicular neurons are inhibited by visual stimuli. **a**, Antidromic activation of a neuron from two loci in ipsilateral SC (blue stars); other stimulation sites (open circles) were ineffective. **b**, The neuron's small contralateral receptive field (shaded red) was in the same hemifield and in spatial register with those at the effective SC stimulation sites (unfilled blue ovals). **c**, The neuron's high spontaneous activity was inhibited by visual stimuli that were moved upwards through the receptive field. Bin width, 10 ms. **d**, Annihilation (open arrow) of antidromic spikes (upper trace) by electrical stimulation (S, filled arrow) triggered by spontaneous spikes during collision tests confirmed antidromic activation (five overlapping traces). **e**, Orthodromic activation (S, filled arrow) indicated input from ipsilateral visual cortex. L, left; R, right. Scale bars, 500 ms (**c**); 1 ms (**d**); 5 ms (**e**).

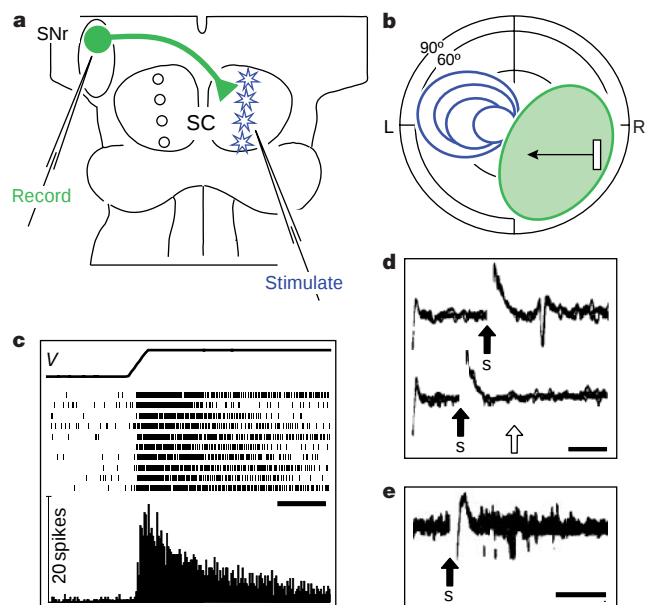


Figure 2 Crossed nigrocollicular neurons are excited by visual stimuli. **a**, Antidromic activation from all sites in the right SC (blue stars) were effective; left SC stimulation sites (open circles) were ineffective. **b**, The neuron's large contralateral receptive field (shaded green) was in the hemifield opposite to those at effective SC stimulation sites (unfilled blue ovals). **c**, Low spontaneous activity was markedly increased by visual stimuli that were moved through the receptive field, and excitation outlasted stimulus presentation. Bin width, 10 ms. **d**, Annihilation (open arrow) of antidromic spikes (upper trace) by electrical stimulation (S, filled arrow) triggered by spontaneous spikes during collision tests confirmed antidromic activation (five overlapping traces). **e**, Orthodromic activation (S, filled arrow) indicated input from ipsilateral visual cortex. L, left; R, right. Scale bars, 500 ms (**c**); 1 ms (**d**); 2 ms (**e**).

the previously recorded crossed neuron (Fig. 4). After control tests, the visual stimulus was concatenated with microstimulation through the contralateral SNr electrode at the peak of the control response. In all such paired recordings ($n = 4$), a brief (0.1 ms), low-intensity (100–300- μ A) pulse delivered through the SNr electrode abolished SC evoked activity for periods of about 30 ms, showing that crossed nigrocollicular neurons exert potent inhibitory influences on the visual responses of neurons in the opposite SC.

These data reveal a striking dichotomy between the physiological properties of crossed nigrocollicular neurons and those generally ascribed to their uncrossed counterparts^{5–8}. They suggest that the

crossed nigrocollicular neurons form one component of complementary ‘push–pull’ pathways that allow the basal ganglia to coordinate neural activity bilaterally in the subcortical circuitry mediating visuomotor behaviours. Working in synchrony, they facilitate the visuomotor activity responsible for acquiring a ‘selected’ target (uncrossed) and simultaneously suppress activity associated with potentially competing distractors (crossed).

These two nigrocollicular populations differed in specific ways that contributed to their functional complementarity. Uncrossed neurons always had comparatively small receptive fields centred in the same hemifield as their SC targets and spatially congruent with the SC receptive fields^{7,8}. Thus, the uncrossed neurons are well suited to disinhibit a specific region of the ipsilateral SC. In contrast, the receptive fields of crossed neurons were significantly larger than their uncrossed counterparts and far larger than those of SC visual neurons. Moreover, the receptive fields of crossed neurons were always centred in the hemifield opposite to those of neurons in the target (that is, contralateral) SC. Antidromic activation of crossed neurons from widespread regions of the opposite SC indicate that these axons might be broadly distributed within the opposite SC.

Despite such physiological differences, our immunohistochemical data indicate that crossed neurons are, like their uncrossed counterparts¹¹, GABA-mediated and inhibit their collicular targets. Moreover, because our data suggest that crossed neurons might establish direct contact with presumptive output neurons, they are appropriately positioned to suppress SC activity and thereby

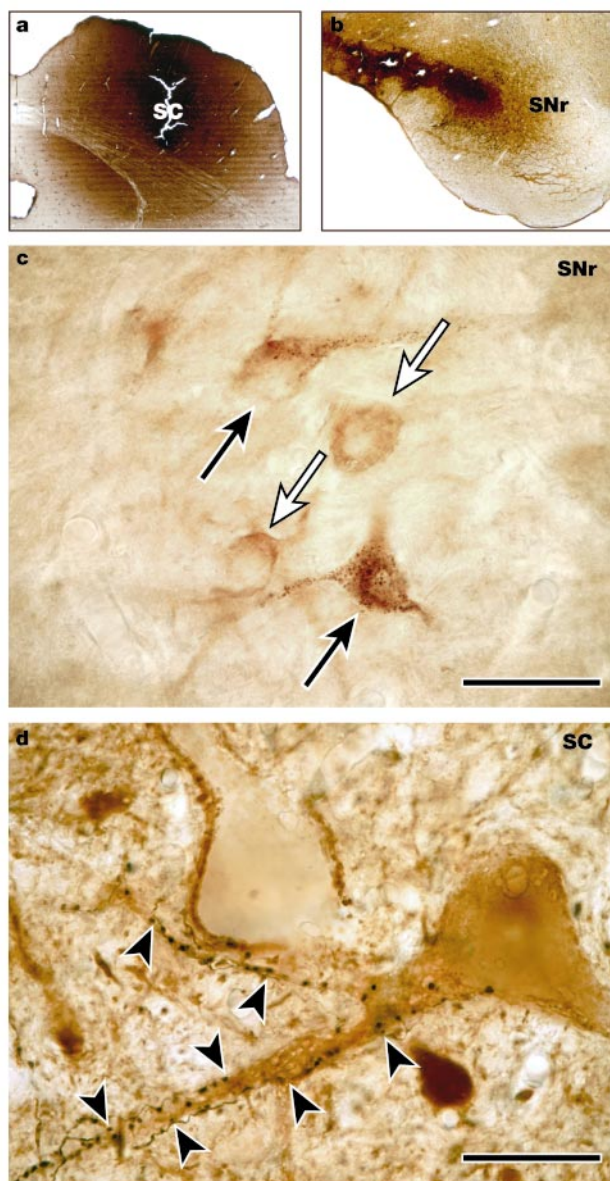


Figure 3 Crossed nigrocollicular neurons are, like their uncrossed counterparts, GABA-mediated and terminate on presumptive output neurons. **a**, wheat-germ agglutinin–horseradish peroxidase (WGA–HRP) deposit in right SC. **b**, BDA deposit in left rostral SNr. **c**, Single-labelled GAD-positive neurons (white arrows) and double-labelled WGA–HRP and GAD-positive neurons (black arrows) in left SNr. All retrogradely labelled neurons contralateral to the injection site were also positive for GAD. **d**, BDA-labelled axonal swellings, indicative of synaptic sites, in close apposition to the primary dendrites (arrowheads) of a presumptive SC output neuron labelled with parvalbumin. Scale bars, 1 mm (**a**, **b**); 50 μ m (**c**, **d**).

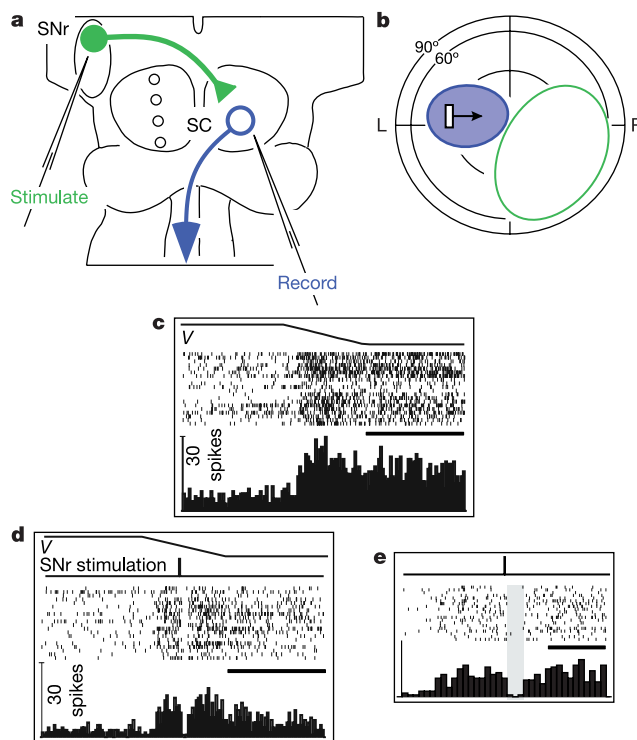


Figure 4 Electrical activation of crossed nigrocollicular neurons inhibits target neurons in contralateral SC. **a**, After analysis of the neuron shown in Fig. 2, stimulating and recording leads were reversed, with visual activity now recorded in the right SC and electrical stimulation delivered at the previous SNr recording site. **b**, The SC neuron’s receptive field (shaded blue) was in the left visual space, whereas the current SNr stimulation site represents right visual space (unfilled green oval; compare with Fig. 2). **c**, Responses of SC neuron to rightward-moving visual stimuli. **d**, Visual stimuli concatenated with brief (0.1-ms) low-intensity (100- μ A) microstimulation delivered through the SNr electrode. Latency to inhibition was 3.9 ms. **e**, Activity at higher temporal resolution revealed that microstimulation for 0.1 ms inhibited visually evoked activity in the SC neuron for about 30 ms. Scale bars, 500 ms (**c**, **d**); 100 ms (**e**). Bin width in **c–e**, 10 ms.

preclude this information from influencing brainstem motor areas. This strategic positioning of crossed nigrocollicular contacts might be a functional necessity, given their comparatively small numbers⁹, and is consistent with our finding that even brief activation of crossed axons results in profound, long-duration suppression of visually evoked activity in the opposite SC.

The significance of such an organizational scheme in the control of visuomotor behaviour is best understood in the light of theories about how gaze shifts are coded by brainstem motor centres. These centres have been shown to compute appropriate movement vectors by averaging the activity from a population of SC neurons³. Although it has been assumed that this neuronal population is restricted to a single SC, specifically the one phasically disinhibited by uncrossed nigrocollicular activity, recent experiments indicate that the activity from both colliculi is integrated in this computation^{19,20}. Thus, actively suppressing neural activity associated with potential distractors in the non-selected SC is just as necessary for achieving the desired gaze shift as facilitating activity associated with the target in the selected SC. This postulate is consistent with previous observations that neurons in the two colliculi fire in a 'push-pull' fashion during spontaneous eye movements: when neurons in one SC become active, those in the other are rendered silent²¹. The crossed nigrocollicular pathways provide an extrinsic substrate for synchronizing intercollicular activity, independently of any contribution from intrinsic SC commissural pathways²². Such interhemispheric coordination might be particularly important in visual search strategies in the natural environment, where the eyes are continually redirected between the hemifields.

The notion that the basal ganglia use global inhibition as well as focal disinhibition is not without precedent. Global inhibition is recognized as an essential component in action selection, in which competing behavioural demands are suppressed while desired programmes are elaborated¹. More specifically, the prevalence of excitatory responses (and, thus, target inhibition) in the globus pallidus, pars interna, during limb movements has been proposed to underlie the coordination of agonist and antagonist musculature during movements²³. Although the lack of excitatory responses in the SNr has prompted suggestions that limb and visuomotor movements are organized differently²³, the present finding (and recent reports of excitation in premotor activity in behaving monkeys^{24,25}) suggests that it is crucial to visuomotor behaviours. These results are consistent with a single operational scheme by which the basal ganglia control the expression of overt behaviours, although the segregation of excitatory responsiveness in crossed nigrocollicular neurons might reflect the biological constraints imposed by the visual system.

The differing responsiveness of uncrossed and crossed neurons to visual stimuli probably reflects the differential convergence of the parallel and antagonistic pathways that course through the basal ganglia²⁶. For example, the phasic inhibition observed in uncrossed neurons is due to the direct striatonigral pathway⁶. Thus, phasic activation of normally quiescent striatonigral GABA-mediated neurons by a visual stimulus will produce the phasic inhibition characteristic of the uncrossed neurons. Although the specific pathway or pathways that produce the phasic excitation of crossed neurons are unknown, it is likely to be due to those transbasal ganglia circuits that ultimately modulate the subthalamus, the source of excitatory input onto SNr neurons²⁷.

One of the intriguing implications of this organizational scheme is to provide a potential physiological basis for the Sprague effect, a paradoxical case of the amelioration of a lesion-induced deficit by a second lesion²⁸. Contralateral visual neglect produced by large unilateral lesions in the visual cortex can be 'reversed' by lesions that affect crossed nigrocollicular neurons^{15,28,29}. According to the present data, eliminating the crossed nigrocollicular pathway removes the widespread inhibition conveyed by these neurons onto the cortically deafferented SC, which presumably reinstates

responsiveness in that SC. This, then, would allow previously ineffectual visual inputs to initiate SC activity and SC-mediated orientation behaviours again. Thus, the amelioration of hemi-neglect can be interpreted as the disruption of an essential component of the circuitry that is normally used by the basal ganglia to coordinate visuomotor activity in the two halves of the neuraxis. This is consistent with the developing understanding that the basal ganglia are critical structures in the manifestation of human hemi-neglect syndromes³⁰. □

Methods

Care of animals

All animal husbandry and experimental procedures were performed in compliance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*, 1996 edition, in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care. Experimental protocols received prior approval by the Institutional Animal Care and Use Committee at Wake Forest University School of Medicine. All efforts were made to minimize the number of animals used and their discomfort.

Physiological techniques

Four adult male cats were prepared for single-unit recording under isoflurane anaesthesia. Two arrays of four epoxylite-coated, tungsten monopolar stimulating electrodes (tip exposure, 150–300 µm; electrode separation, 1 mm) were implanted in the intermediate laminae of each SC for antidromic activation. The array spanned the entire rostrocaudal extent of the SC, with each electrode's tip resting within the intermediate grey layer. The final position of the array was determined by recording. A similar array was implanted in the medial and lateral banks of the left lateral suprasylvian cortex for orthodromic activation. Maximum stimulation intensities never exceeded 300 µA. Electrode access to the SNr was provided by a recording chamber, and the head was held by an atraumatic head-holder.

For chronic recording, animals were anaesthetized with ketamine hydrochloride (20 mg kg⁻¹, intramuscular) and acepromazine maleate (0.5 mg kg⁻¹, intramuscular), intubated, paralysed with pancuronium bromide (induction 0.3 mg kg⁻¹, intravenous), and artificially ventilated. Anaesthesia was maintained by a constant infusion of ketamine (5 mg kg⁻¹ h⁻¹) and pancuronium (0.1 mg kg⁻¹ h⁻¹). Pupils were dilated with 1% atropine sulphate, and the nictitating membranes and eyelids were retracted with 2.5% phenylephrine hydrochloride. The refractive state of each eye was measured by direct ophthalmoscopy, and contact lenses of the appropriate powers were fitted to focus the eyes on a translucent Plexiglas hemisphere positioned in front of the animal. The positions of the optic disc and area centralis of each eye were plotted onto the dome by means of reverse ophthalmoscopy. Neurons were selected by antidromic activation and confirmed by a 'collision test'. Because the initial search strategy of examining every isolated neuron encountered during a penetration produced a very low yield of crossed neurons (the anatomic ratio of uncrossed to crossed neurons is more than 12:1 (ref. 9)), antidromic activation from the contralateral SC was used as the primary search strategy to maximize the sample of crossed neurons. The numbers in each category reported in this study therefore reflect a biased search strategy and are an overestimate of the relative frequency of crossed neurons. However, in all cases each neuron in the sample was tested for activation from all electrodes in both SCs.

Receptive-field mapping and initial qualitative evaluation of neuronal response properties were conducted by using spots or bars of light projected from a hand-held pantoscope. Only neurons with receptive-field centres clearly within one hemifield or the other (that is, 10° or more from area centralis) were included in this study. For quantitative assessment, light stimuli of various shapes and sizes were projected onto the hemisphere by means of an electronically controlled, galvanometer-driven mirror system. Stimuli were presented repeatedly ($n = 10$ –20 times per test, interval 6 s). Neuronal activity was acquired with Spike2 software (Cambridge Instruments) and analysed offline with Matlab (MathWorks) and Excel (Microsoft).

Anatomical techniques

For retrograde analysis of nigrotectal neurons, pressure injections of 0.065 µl 2% wheat-germ agglutinin–20% horseradish peroxidase (Sigma) were made with a 1-µl Hamilton microsyringe into the SC of four isoflurane-anaesthetized cats. Two of the animals had been used previously for electrophysiological experiments. After 24 h survival, animals were perfused with 0.9% saline followed by 4.0% paraformaldehyde–0.1% glutaraldehyde fixative in 0.1 M phosphate buffer, pH 7.2. The brains were stereotactically blocked, then removed and refrigerated overnight at 4 °C in 0.1 M sodium phosphate buffer. Coronal sections were cut on a vibratome at 60 µm thickness and placed in 0.1 M sodium phosphate buffer. Alternate sections were reacted with tetramethylbenzidine as the chromogen. Adjacent sections were reacted with cobalt-intensified diaminobenzidine and then processed for anti-GAD antibody (Sigma) with Vectastain ABC (Sigma).

For anterograde analysis of nigrotectal terminals, pressure injections of 1.0 µl 5% biotinylated dextran (Sigma) were made into the rostral SNr of four animals. After survival for 5 days, animals were perfused with 0.9% saline followed by 4.0% paraformaldehyde–0.1% glutaraldehyde fixative in 0.1 M phosphate buffer, pH 7.4. Adjacent sections were reacted for biotinylated dextran amine (BDA), then processed for parvalbumin (Sigma) immunohistochemistry with Vectastain ABC. Analysis and

photomicroscopy were conducted with an Olympus BX50 and a Wild M400 Photomakroskop. Images were captured with a Spot RT slider digital camera (Diagnostic Instruments), and image files were processed with Photoshop (Adobe) and Canvas (Deneba) software. Images for publication were corrected for contrast and brightness.

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Mapping multiple features in the population response of visual cortex

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Stimulus features such as edge orientation, motion direction and spatial frequency are thought to be encoded in the primary visual cortex by overlapping feature maps arranged so that the location of neurons activated by a particular combination of stimulus features can be predicted from the intersections of these maps^{1–8}. This view is based on the use of grating stimuli, which limit the range of stimulus combinations that can be examined. We used optical imaging of intrinsic signals⁹ in ferrets to assess patterns of population activity evoked by the motion of a texture (a field of iso-oriented bars). Here we show that the same neural population can be activated by multiple combinations of orientation, length, motion axis and speed. Rather than reflecting the intersection of multiple maps, our results indicate that population activity in primary visual cortex is better described as a single map of spatiotemporal energy.

Orderly relationships between overlapping feature maps in the visual cortex are thought to ensure that all combinations of stimulus features are represented uniformly across the visual field^{2–8}. In this conception, a specific combination of stimulus features (such as a vertical stimulus of high spatial frequency moving to the right) is represented by a unique pattern of population activity that corresponds to appropriate intersecting regions of different feature maps. This view is based on the analysis of cortical responses to drifting gratings in which the range of motion and spatial frequency cues is limited to those that vary along an axis orthogonal to the grating's orientation^{10–12}. As a result, it has not been possible to examine interactions between motion, orientation and spatial frequency that are common in the visual environment. To decouple these stimulus features, we used texture stimuli composed of iso-oriented line segments^{13,14} (see Supplementary Fig. 1a). Patterns of cortical activity evoked by various combinations of line orientation and axis of motion were assessed by optical imaging of intrinsic signals and by electrophysiological recordings; in addition we explored how these patterns were altered by changes in line length and motion speed.

Texture stimuli produced strong and reliable activation of visual cortical neurons, evoking a spatial pattern whose structure and periodicity resembled that found with square-wave grating stimuli (Fig. 1). For texture stimuli moved along axes perpendicular to the line elements, the activation patterns were statistically indistinguishable from those evoked by gratings of the same orientation (Fig. 1a–c). For example, a 45° texture yielded a population response (Fig. 1c; see Methods) that peaked near 45° and was similar to that found for a 45° grating. Across the seven animals examined, the peak and width of the population response for textures with orthogonal motion (peak = 42° ± 3° (mean ± s.e.m.), half-width at half-height = 46.7° ± 3.6°) were not significantly different from that for gratings (peak = 41° ± 1°, half-width = 40° ± 2.3°; *P* = 0.7 and 0.1 respectively, *t*-test). The tuning functions derived from the optical responses were also well fitted by gaussian functions (average *R*² for gratings = 0.91 ± 0.02; for textures *R*² = 0.90 ± 0.03).

In contrast, movement of the textures along non-orthogonal axes produced population responses that differed significantly from those found in response to gratings matching the orientation of the texture bars. For example, a 45° clockwise shift in the axis of motion, without altering the orientation of the line elements (45°),

changed the distribution of population activity so that it peaked near 20° rather than 45° (Fig. 1f). This shift can also be appreciated qualitatively in the difference images (compare Fig. 1a and d). In a symmetrical fashion, a 45° anticlockwise shift in the axis of motion changed the population response such that its peak was at 75° (Fig. 1i; compare Fig. 1a and 1g). Despite the fact that the images in Fig. 1d, g were obtained with textures consisting of 45° bars, the activity patterns were very different from each other ($R^2 = 0.01$; see Methods), indicating a strong effect of axis of motion. Across the seven animals examined, a 45° offset between the orientation of the elements and the axis of motion yielded an average shift in the peak of the population response of $39^\circ (\pm 3^\circ)$. Correspondingly smaller shifts in the peak of the population response were found for a 22.5° offset in the axis of motion ($20^\circ \pm 7^\circ$).

Despite these shifts, the pattern of population activity for non-orthogonal motion still resembled that found with grating stimuli. The average half-width of the population response for textures moving non-orthogonally was quite similar to the orthogonal texture or the grating response ($39.1^\circ \pm 2.7^\circ$), as was the average R^2 value for the best-fit gaussian function (0.94 ± 0.02). However,

the patterns were now more similar to those evoked by gratings whose orientation differed from the orientation of the texture elements. For Fig. 1d–f the pattern resembled that evoked by a more acute grating angle ($R^2 = 0.46$ between difference images in Fig. 1d and e), whereas for Fig. 1g–i it resembled that evoked by a less acute grating angle ($R^2 = 0.7$ between difference images in Fig. 1g, h). In both cases the patterns are quite different from those evoked by an orientation-matched grating ($R^2 = 0.25$ between difference images in Fig. 1d and b and 0.16 between difference images in Fig. 1g and b). These results suggest that the iterated pattern of activity resulting from the presentation of a grating stimulus, which has been thought to represent the orientation of the grating, can be elicited by a range of different orientation and axis-of-motion combinations made possible by the use of texture stimuli (see Supplementary Fig. 1b).

The dissociation of the activity patterns for textures from patterns evoked by their orientation-matched gratings is further emphasized under conditions in which the length of the texture line segments is altered without changing either the line orientation or the axis of motion. All experiments shown in Fig. 1 were performed by keeping the bar length fixed at 6° of visual angle. Increasing the line length from 2° to 10° without changing the line orientation (45°) or the axis of motion (horizontal) produced a systematic change in the population response (Fig. 2a and Supplementary movie 1). Quantification of the population response shows the progressive shift in the peak with increasing stimulus length, from a peak of 87° for short line segments to 56° for long line segments. Similar results were obtained in all eight animals tested (Fig. 2c). The average change in the peak with change in line length from 2° to 10° was 31° and this difference was highly significant ($P < 0.001$, one-way analysis of variance). Because changing the line length changes the spatial frequency characteristics of the stimulus (see ref. 12), it is possible that these shifts in the pattern of population activity could be attributable to the orderly mapping of spatial frequency^{5,6,15}. However, if this were so, similar changes in the population response to a change in line length should be found for a motion of the texture along the axis orthogonal to the orientation of the elements. Although increasing the line length affected the magnitude of the population response, there was no systematic change in the peak of the response for orthogonal motion (Fig. 2b). This result also excludes the possibility that the shifts in the distribution of population activity with line length can be explained by a systematic mapping of length preference such as might result from variations in length summation properties or end-stopping^{16,17}.

From the results presented so far, one could infer that a given pattern of activity can be elicited by a range of stimuli that are composed of different combinations of line orientation, axis of motion and length. The population response to texture stimuli therefore cannot encode a unique combination of stimulus features, nor can it simply correspond to the intersection of multiple feature maps. Instead, a description of the population response in terms of the tuning of individual neurons to a restricted range of spatial (along two dimensions) and temporal frequencies^{12,18–21} might be a more appropriate framework for interpreting these results. The short line segments of the texture stimuli possess energy at many orientations in the Fourier domain^{18,22} (that is, they are characterized by a broad spatial frequency spectrum). Modulation of the neural response to all Fourier components could explain why the tuning of the population response does not match that predicted from the orientation of the texture elements²¹. When the stimulus moves, each Fourier component moves at a different speed depending on its orientation relative to the direction of motion of the stimulus (see Supplementary Fig. 2). The component whose speed is optimal given the temporal tuning properties of neurons in the primary visual cortex (V1) determines the peak of the active population. Thus, a strong test of the ability of spatiotemporal

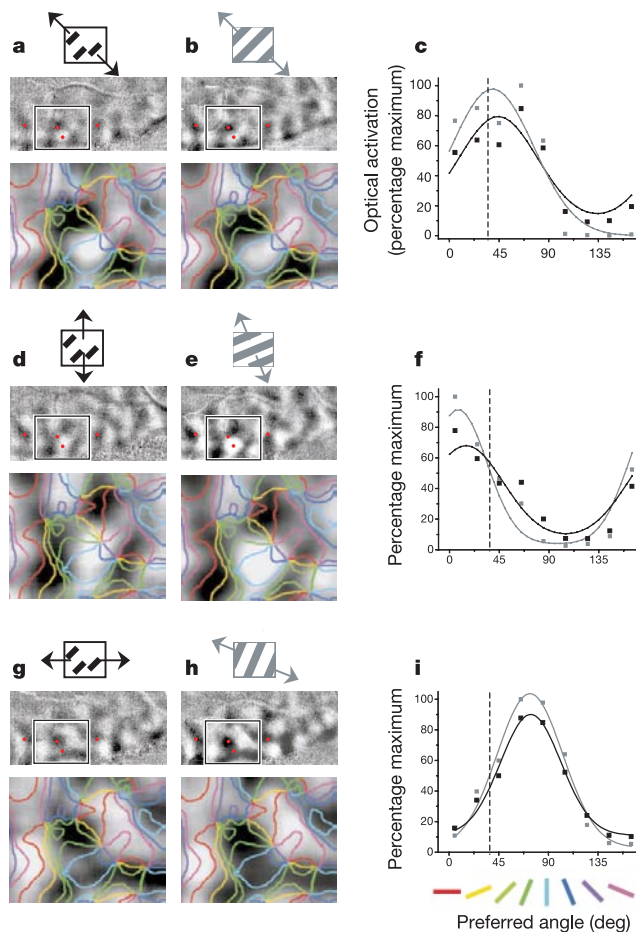


Figure 1 Systematic shifts in the population response induced by changes in axis of motion. Each panel contains texture (a, d, g) or grating (b, e, h) difference images, with insets showing iso-orientation contours derived from the grating angle map overlaid on the boxed region of interest (see angle colour key below graph, inset width = 1.7 mm). The red dots are placed over identical regions of each image to facilitate comparison. Graphs quantify the population response (c, f, i: black curve, texture response; grey curve, grating response). The dashed vertical line is placed at the same position on the x-axis to compare the relative shift between the curves. a–c, Orthogonally moving textures and gratings of the same orientation evoke similar responses. d–f, A 45° clockwise shift in texture motion evokes a response similar to a 22° grating. g–i, A 45° anticlockwise shift in texture motion evokes a response similar to a 67° grating.

frequency tuning to account for the population response is whether the activity patterns evoked by texture stimuli are sensitive to the speed of texture motion^{23,24} (but see ref. 25).

This prediction was tested by using a texture composed of 45° oriented bars of 4° length moving horizontally at 10° s⁻¹, 50° s⁻¹ and 100° s⁻¹. Changes in speed resulted in significant shifts in the locus of activity (Fig. 3a). These changes correspond to a shift in the

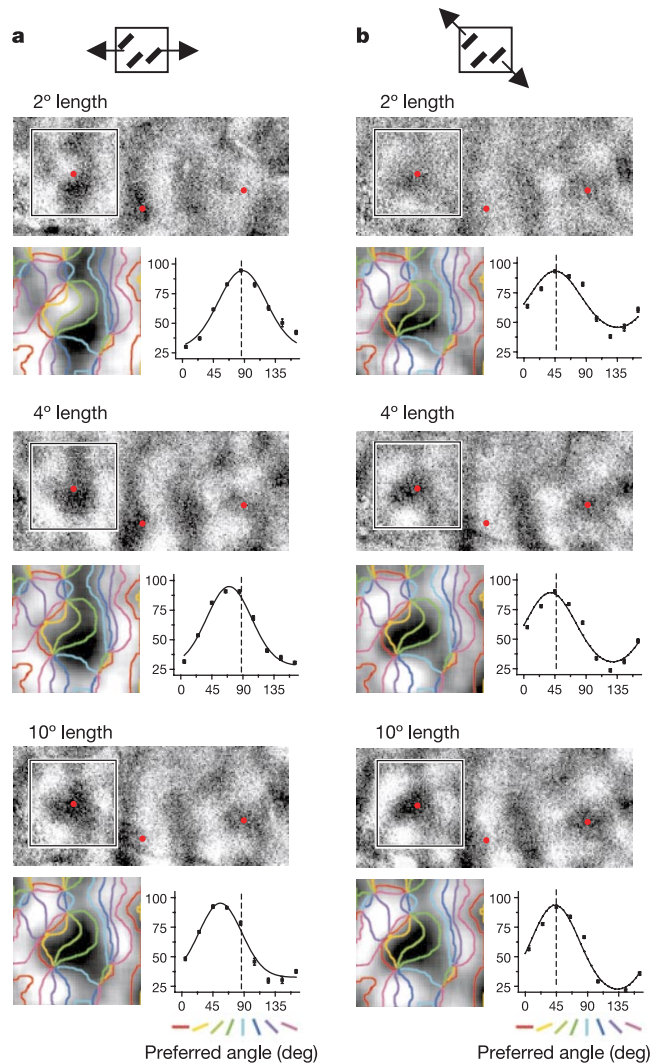


Figure 2 Systematic shifts in the population response induced by changes in bar length. **a**, Difference images, contour overlays (inset width = 1.2 mm) and population response profiles for texture stimuli of three lengths (2°, 4° and 10°) moved non-orthogonally. Increasing the bar length shifted the activity pattern and the peak of the response from 87° to 70° to 56° (from blue contours to green–yellow contours; see Supplementary movie 1). **b**, Lack of shifts with orthogonal motion. **c**, Summary of length-induced shifts with non-orthogonal textures ($n = 8$ animals; results are means $\pm$ s.e.m.); peaks for 2°, 4° and 10° lengths are 90°, 74° and 59°, respectively.

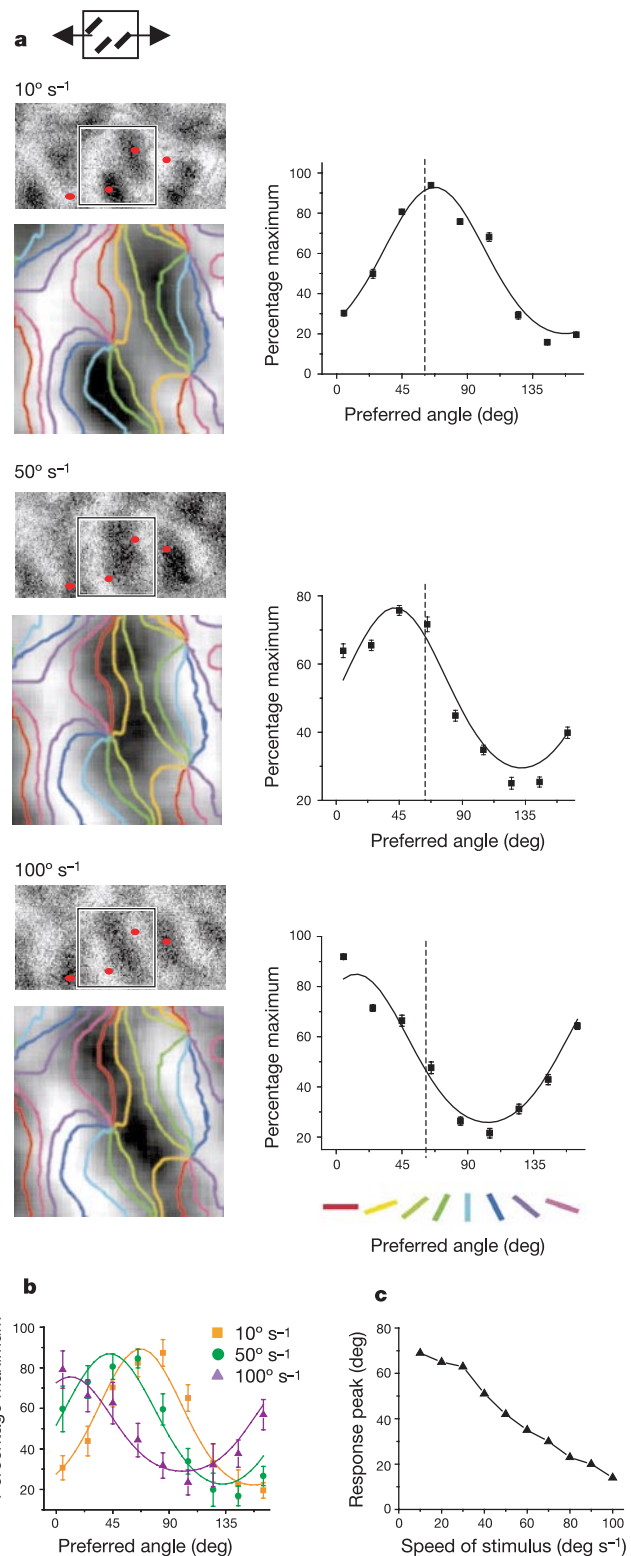


Figure 3 Shifts in the population response induced by changes in stimulus speed. **a**, Difference images, contour overlays (inset width = 1.3 mm) and population response profiles obtained with 45° texture stimuli moving horizontally at three speeds: 10° s⁻¹ (peak = 69°), 50° s⁻¹ (peak = 42°) and 100° s⁻¹ (peak = 14°). **b**, Average population responses (means $\pm$ s.e.m.) for three speeds ($n = 7$ animals); the peak shifts from 73° at low speed to 47° at intermediate speed to 17° at high speed. **c**, Peaks of the population responses obtained with 10 different speeds (10–100° s⁻¹ at 10° s⁻¹ intervals; see Supplementary Fig. 3).

peak of the population response of 55° as the speed increased from 10° s^{-1} to 100° s^{-1} . Similar results were found for all seven animals tested (Fig. 3b, change in peak with speed was significant at $P < 0.001$, one-way analysis of variance). In one animal we examined the population response to 10 speeds ranging from 10° s^{-1} to 100° s^{-1} at 10° intervals (see Supplementary Fig. 3 and Supplementary movie 2). The peak of the response varied linearly over a 60° range as speed increased (Fig. 3c; R^2 of linear fit = 0.99).

If these changes in population activity resulted from the orderly mapping of preferences for speed or temporal frequency^{15,26}, similar shifts should be found with grating stimuli. This did not occur (Supplementary Fig. 4a, c). The average peaks of response for vertical gratings drifting at 20° s^{-1} ($90.3^\circ \pm 0.7^\circ$) and at 100° s^{-1} ($89.7^\circ \pm 1^\circ$) were not significantly different ($P = 0.6$, t -test, $n = 6$ animals). In contrast, and consistent with the spatiotemporal energy framework, patterns of population activity induced by drifting random-dot stimuli showed pronounced changes with speed (Supplementary Fig. 4b₁, b₂, d), the average shift in peak of

the population response from low to high speed, for eight animals, was $89.2^\circ \pm 4.2^\circ$. Furthermore, speed-induced changes in population response to the textures described above varied systematically with line length. Because an increase in line length narrows the power spectrum of the stimulus²¹, it is predicted that the magnitude of the speed-induced shift will decrease with an increase in length. In two cases, for texture stimuli 2° long, the average shift in population tuning with change in speed from 10° s^{-1} to 100° s^{-1} was $86^\circ \pm 1.75^\circ$, for 4° textures it was $57^\circ \pm 2.7^\circ$ and for 10° textures the shift was only $22^\circ \pm 8.15^\circ$.

If tuning to spatiotemporal frequency explains the population response, the activity of single neurons should display length- and speed-dependent shifts comparable to those seen with optical imaging. Shifts in the tuning of single and multiple units elicited by the same texture and dot stimuli that were used for imaging analysis were similar in magnitude and direction to those found in the population response (Fig. 4). For example, changing the length of the line segments in textures undergoing non-orthogonal motion produced significant shifts in the preferred angle of stimulus motion (Fig. 4a, b). Moreover, similar shifts were obtained with single bars moving non-orthogonally, showing that the effect did not depend on the use of texture stimuli (Supplementary Fig. 5). Changing the speed of motion of dots (Fig. 4c, d) and textures (Fig. 4e, f) also produced consistent and significant shifts in the preferred direction of stimulus motion for single units in V1. Similar shifts were observed for both direction-tuned and non-direction-tuned neurons.

These results are difficult to reconcile with the view that V1 comprises multiple maps of orientation, direction and spatial frequency whose intersections signal the presence of particular feature combinations^{2–8}. Our data show that the spatial distribution of active cortical columns does not by itself specify the exact combination of stimulus features present in the scene, because many different combinations activate similar populations of neurons. Nevertheless, the spatial pattern of population activity is very sensitive to small changes in each of the properties that were tested and these changes are highly reproducible: a particular change in stimulus configuration produced similar shifts in population activity across all animals in our sample. These results indicate that the columnar architecture of V1 represents a spatiotemporal transform in which multiple features are combined. Rather than multiple maps of different stimulus features, we suggest that the mapping of one property—orientation in space-time^{27,28}—accounts for both the orderly shifts in patterns of activity and the conflation of different stimulus features in the population response. □

Methods

Ethical approval

All experimental procedures were approved by the Duke University Institutional Animal Care and Use Committee and were performed in compliance with guidelines published by the National Institutes of Health (USA).

Surgical procedures

Animals were prepared for optical imaging as described in ref. 29. In brief, ferrets (postnatal days 45–65) were anaesthetized with a mixture of ketamine and xylazine. A hole was made in the cranium over the visual cortex, the dura was reflected and a chamber made of agar and a glass coverslip was mounted over the brain. Ferrets were paralysed with rocuronium bromide ($0.3 \text{ mg kg}^{-1} \text{ h}^{-1}$) either intraperitoneally or intravenously and respired with a mixture of $\text{N}_2\text{O}:\text{O}_2$ (2:1) supplemented with 0.5–1.5% halothane. Expired CO_2 was maintained at 4% and body temperature was maintained at 37°C .

Optical imaging and visual stimulation

Optical imaging of intrinsic signals was performed with an enhanced video acquisition system (Optical Imaging) as described previously^{9,29}. Stimuli were full-field ($80^\circ \times 60^\circ$) high-contrast square-wave gratings, full-field textures, coherently drifting random-dot patterns or single bars. Dots were 0.25° in diameter with a density of ~ 10 dots per 10 square degrees. Textures were pseudo-randomly placed high-contrast square-wave bars whose length, width, density and orientation could be varied. Bar density was decreased while increasing the length to control for total luminance. Bar width was always 1° while length was varied as described above. Difference images were generated by subtracting the optical responses to the presentation of a particular orientation or axis of motion from its orthogonal angle or axis. Thus, a 90° grating image was produced by subtracting the

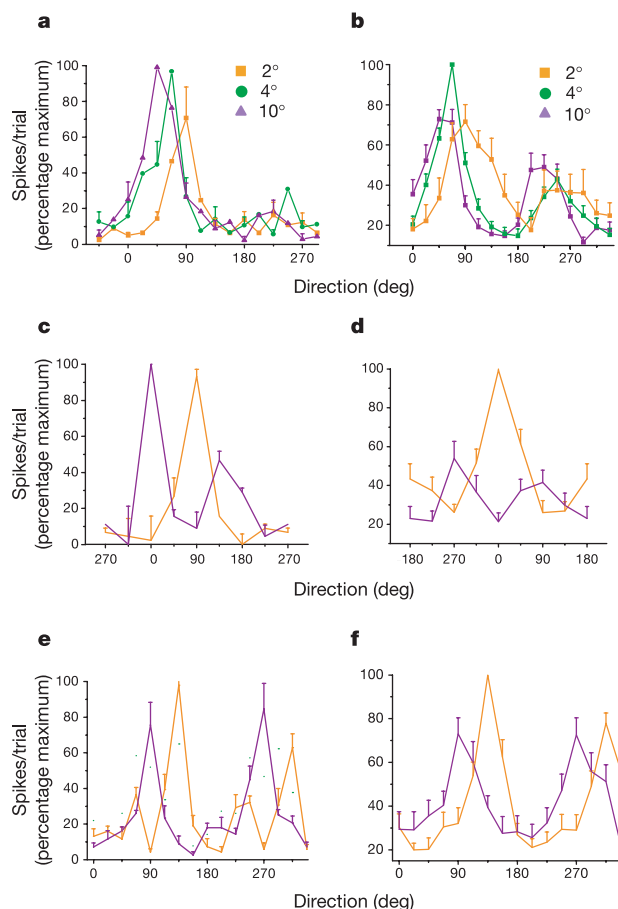


Figure 4 Tuning shifts seen with imaging occur at the single unit level. **a**, Tuning curves for a single unit obtained with non-orthogonally moving textures. The preferred angle shifts as the bar length increases. **b**, Average tuning curves for all recorded units for three stimulus lengths ($n = 11$ units for 2° length, 23 units for 4° length and 20 units for 10° length). **c**, Tuning curves for a single unit obtained with random-dot patterns drifting at 20° s^{-1} (orange line) and 100° s^{-1} (violet line). **d**, Average tuning curves obtained with random dots for the same two speeds ($n = 13$ units). **e**, Tuning curves for a single unit obtained with non-orthogonally moving textures drifting at 20° s^{-1} and 100° s^{-1} . **f**, Average tuning curves obtained with non-orthogonally moving textures at the same two speeds ($n = 13$ units). All results are means and s.e.m.

response to a 90° grating from that to a 0° grating. For stimuli undergoing non-orthogonal motion, both the orientation and the axis of motion were different by 90° between the subtracted stimuli. For example, the response to a texture with 45° oriented bars moving horizontally was subtracted from the response to a texture with 135° oriented bars moving vertically. Results obtained with difference imaging were verified with single-condition imaging by subtracting the response to a blank screen from the stimulus-driven response (see Supplementary Fig. 6).

Optical map analysis

To interpret the patterns of activity evoked by various stimulus configurations, we developed a population response profile that captured the relative activation of each pixel in the region of interest and expressed these values in terms of the pixel's preferred orientation assessed with gratings. Grating images obtained for four to eight orientations were low-pass and high-pass filtered and vector-summed to produce referent grating-angle maps*. To compute a population response profile for a given stimulus, the difference image for that stimulus was assigned a threshold at the mean grey value and each pixel above the threshold was weighted (greyscale value minus threshold). On the basis of each pixel's preferred grating orientation, the weighted pixel values were summed into nine 20° angle bins (from 0° to 180°) and normalized to the highest count across conditions (for example, across length or speed). The resulting histograms were fitted with gaussian functions and the peak and width of the response were determined along with the R^2 value of the best-fit gaussian function. For direct comparison between any two images, the two-dimensional correlation coefficient (R) between the two (high-pass and low-pass filtered) images was determined with the Matlab Image Processing Toolbox (Mathworks, Natick, Massachusetts). This value was then squared and reported as the more intuitive coefficient of determination (R^2).

Difference images shown in figures were high-pass filtered and a region of interest was selected for demonstration purposes. The region of interest was selected as the region of the image that showed both a strong response and a representative shift with change in stimulus conditions. Contour overlays were prepared by superimposing iso-orientation contours from a referent grating-angle map over a filtered and re-clipped (to ± 2 s.d.) texture, dot or grating difference image.

Electrophysiology

Single and multiple units were recorded extracellularly from V1 with a tungsten microelectrode (impedance 8–14 M Ω). Action potentials were recorded from the superficial layers (<400 μ m) and discriminated by using Spike2 software (Cambridge Electronic Design, Cambridge, UK). Orientation and direction tuning functions were obtained for each site by panning a bar (40° $\times$ 0.4°) across the receptive field. Tuning curves for textures or dots were obtained by panning full-field textures (at eight orientations) or a field of dots in 16 directions (at 22.5° intervals). For non-orthogonal motion, the offset between bar orientation and direction of motion was 45°. For the single-bar experiments, the centre and size of the minimal discharge field were determined by using a small orthogonally drifting bar. A square-wave bar of varying length was then moved non-orthogonally such that it always passed through the centre of the receptive field. In some cases, 3–10 speeds were randomized within the direction tuning experiment. Action potentials were counted for the entire stimulus duration (1–2 s). Offline spike sorting and tuning analysis was performed with custom-written Spike2 software.

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Cyclic AMP/GMP-dependent modulation of Ca²⁺ channels sets the polarity of nerve growth-cone turning

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Signalling by intracellular second messengers such as cyclic nucleotides and Ca²⁺ is known to regulate attractive and repulsive guidance of axons by extracellular factors^{1,2}. However, the mechanism of interaction among these second messengers in determining the polarity of the guidance response is largely unknown. Here, we report that the ratio of cyclic AMP to cyclic GMP activities sets the polarity of netrin-1-induced axon guidance: high ratios favour attraction, whereas low ratios favour repulsion. Whole-cell recordings of Ca²⁺ currents at *Xenopus* spinal neuron growth cones indicate that cyclic nucleotide signalling directly modulates the activity of L-type Ca²⁺ channels

(LCCs) in axonal growth cones. Furthermore, cGMP signalling activated by an arachidonate 12-lipoxygenase metabolite³ suppresses LCC activity triggered by netrin-1, and is required for growth-cone repulsion mediated by the DCC–UNC5 receptor complex⁴. By linking cAMP and cGMP signalling and modulation of Ca^{2+} channel activity in growth cones, these findings delineate an early membrane-associated event responsible for signal transduction during bi-directional axon guidance.

A gradient of netrin-1 induces chemoattraction of *Xenopus* spinal neuron growth cones by means of the netrin-1 receptor deleted in colorectal cancer (DCC)⁵, whereas overexpression of another netrin-1 receptor (UNC5) in these neurons causes netrin-1 to induce chemorepulsion through the DCC–UNC5 receptor complex⁴. In neurons not overexpressing UNC5, the DCC-mediated attraction is converted to repulsion when cAMP signalling is blocked by the membrane-permeable cAMP antagonist Rp-cAMPS or the protein kinase A (PKA) inhibitor KT5720 (ref. 6). The association between cyclic nucleotide signalling and DCC–UNC5-mediated repulsion is not known.

We therefore examined the relationship between cAMP and cGMP signalling on DCC-mediated attraction in control neurons, and on DCC–UNC5-mediated repulsion in UNC5H2-overexpressing neurons. In control neurons, bath application of KT5720 (200 nM) converted DCC-mediated attraction to repulsion (Fig. 1a, c)⁶, and this repulsion was abolished by the membrane-permeable cGMP analogue 8-bromo-cGMP (8-Br-cGMP, 20 μM) (Fig. 1a, c). Bath-application of 8-Br-cGMP or *S*-nitroso-*N*-acetylpenicillamine (SNAP, 300 μM), a nitric oxide donor known to

elevate the cytosolic cGMP level⁷, completely converted DCC-mediated attraction to repulsion (Fig. 1a, right panel, c). However, at a higher concentration of 8-Br-cGMP (100 μM), we found that the attractive response was not affected, consistent with an earlier report⁸ (Supplementary Information). Thus, the modulatory effects of 8-Br-cGMP seem to be dose dependent, and netrin-1-induced turning responses might be regulated by both cAMP and cGMP. For DCC–UNC5-mediated repulsion, bath application of KT5720 abolished the repulsion induced by a netrin-1 gradient (Fig. 1b, c), although it had no significant effect on semaphorin 3A (Sema3A)-induced repulsion⁸ (Fig. 1c). Furthermore, application of the protein kinase G (PKG) inhibitor KT5823 (750 nM) converted DCC–UNC5-mediated repulsion to attraction (Fig. 1b, c), whereas DCC-mediated attraction in control neurons was not significantly affected (Fig. 1c). Taken together, these results indicate that cGMP signalling has a major regulatory role in netrin-1-induced repulsion, whereas cAMP signalling modulates both DCC- and DCC–UNC5-mediated attraction and repulsion, respectively. Because cAMP signalling did not affect neuropilin-1 (NP-1)-mediated repulsion induced by Sema3A (Fig. 1c and ref. 9), the dependence on cyclic nucleotide signalling pathways might differ among different types of repulsive signals.

To address the relative roles of cAMP- and cGMP-dependent pathways in growth-cone guidance by netrin-1, we systematically varied the ratio of the membrane-permeable cyclic nucleotide analogues Sp-8-Br-cAMPS and 8-Br-cGMP, while maintaining their total concentration at 10 μM . As shown in Fig. 1d, as the ratio of the concentration of [Sp-8-Br-cAMPS]/[8-Br-cGMP]

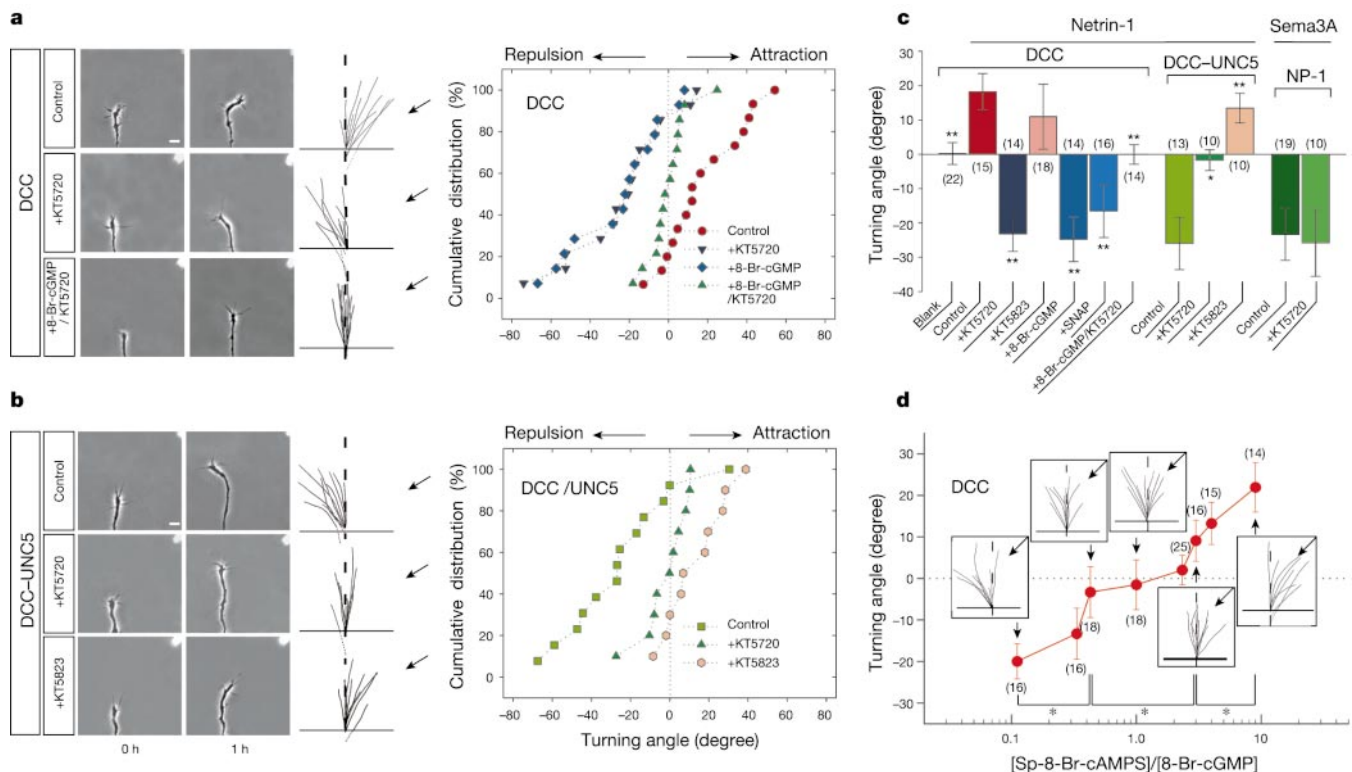


Figure 1 cAMP/cGMP signalling-dependent bi-directional growth-cone turning induced by netrin-1. **a, b**, Growth cones of DCC-expressing⁵ (DCC) and UNC5H2-overexpressing⁴ (DCC–UNC5) neurons before (0 h) and after (1 h) exposure to a netrin-1 gradient (arrows)⁴ under various conditions (left panels). Traces depict the trajectories of neurite extension after each assay. The right panels show the distribution of turning angles for all neurons examined. Scale bars, 10 μm . **c**, Average turning angles for each assay. The column labelled ‘blank’ indicates that netrin-1 was omitted in the pipette. Significant differences

from the control group are indicated (asterisk, $P < 0.05$; double asterisk, $P < 0.01$; Mann–Whitney *U*-test). **d**, Average turning angles of DCC neurons after exposure to a netrin-1 gradient at different [Sp-8-Br-cAMPS]/[8-Br-cGMP] ratios in the bath. Samples of neurite trajectories are shown (insets). Significant differences between the ratios are indicated (asterisk, $P < 0.05$; Kolmogorov–Smirnov test). Numbers in parentheses indicate the total number of growth cones examined. Error bars indicate standard error of the mean (s.e.m.).

decreased, growth-cone turning induced by the netrin-1 gradient shifted from attraction to repulsion. A significant shift in turning behaviour was observed when one cyclic nucleotide was at least threefold in excess of the other, whereas a ninefold excess of [Sp-8-Br-cAMPS] or [8-Br-cGMP] ensured a significant attractive or repulsive response, respectively (Fig. 1d, see also Supplementary Information). The turning responses correlate with the ratio of cAMP and cGMP signalling: a high [cAMP]/[cGMP] ratio favours attraction, whereas a low ratio favours repulsion. Such second-messenger-dependent bi-directional behaviour is reminiscent of the dependence of synaptic potentiation/depression on stimulus frequency or postsynaptic Ca^{2+} levels, as described by the Bienenstock–Cooper–Munro theory of synaptic plasticity^{10–12}. The dependence of growth-cone turning on the ratio of [cAMP] to [cGMP] could also account for the observations described earlier on DCC- and DCC–UNC5-mediated turning responses, and are consistent with the modulation of signal transduction pathways by these cyclic nucleotides.

How do cAMP and cGMP signalling pathways modulate netrin-1

signals? We have demonstrated previously that in *Xenopus* neurons, netrin-1-induced attraction requires Ca^{2+} entry through voltage-dependent Ca^{2+} channels (VDCCs) including LCCs², which are known to be activated by cAMP but inactivated by cGMP signalling pathways^{13,14}. Recent studies of *Xenopus* neurons demonstrated that spontaneous Ca^{2+} spikes, which depend on Ca^{2+} entry through VDCCs and Ca^{2+} release through ryanodine receptors (RyRs)¹⁵, correlate with transient changes in the intracellular concentration of cAMP¹⁶. Therefore, we measured Ca^{2+} channel activity by whole-cell voltage-clamp recordings of growth cones (Fig. 2a). In control growth cones, netrin-1 induced a marked increase in the amplitude of L-type Ca^{2+} currents, as revealed by the magnitude of nimodipine-sensitive (20 μM) inward currents observed in response to depolarizing voltage steps¹⁷ in the presence of tetrodotoxin (TTX), caesium ions (Cs) and tetraethylammonium (TEA) (Fig. 2b, c, e; see Methods). These increases in Ca^{2+} currents were not observed at the soma in the presence of a netrin-1 gradient towards the growth cone (Fig. 2b; see also Supplementary Information). In contrast to that found in control neurons, netrin-1 significantly decreased Ca^{2+}

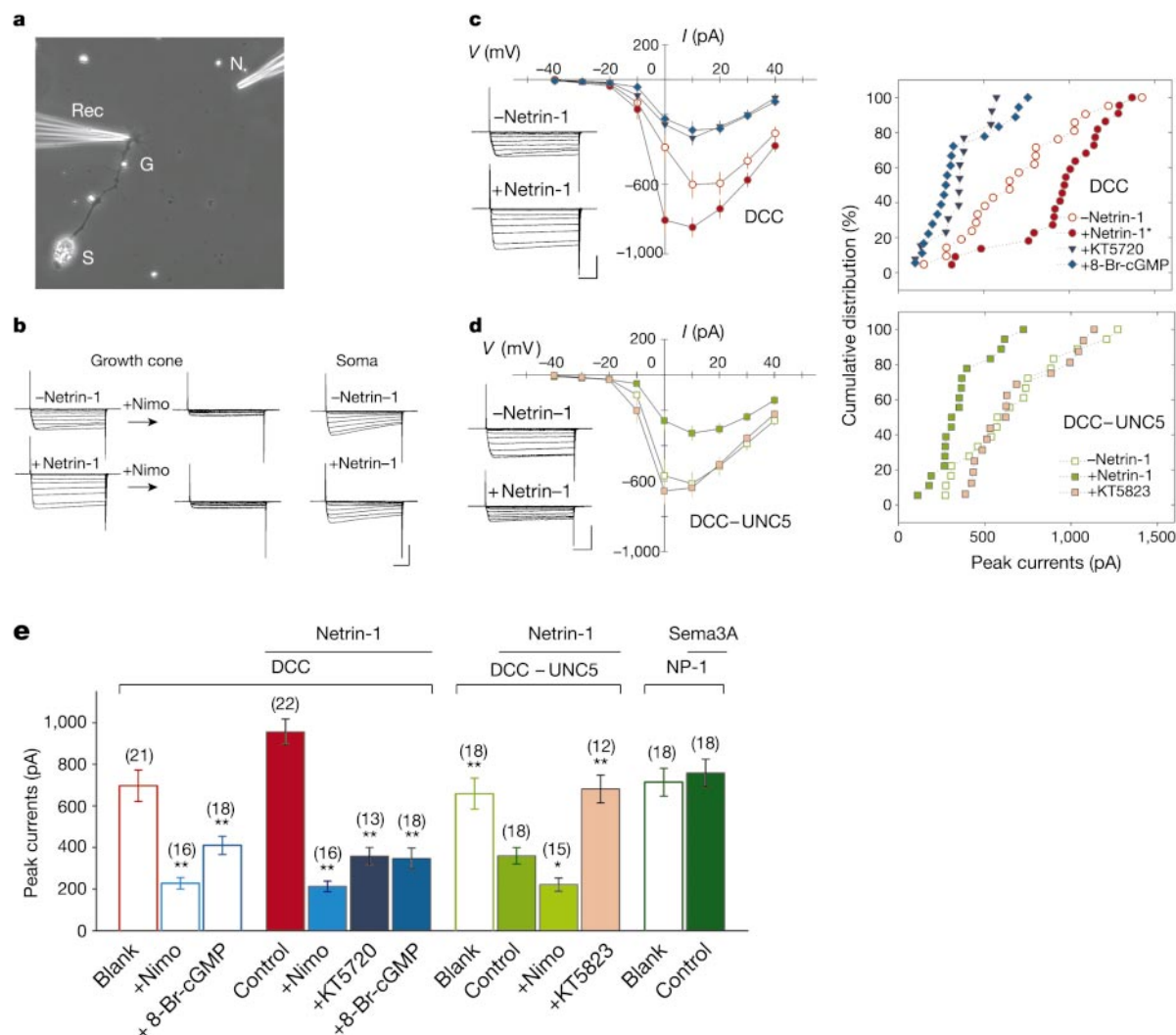


Figure 2 cAMP/cGMP signalling-dependent modulation of LCC activity induced by netrin-1. **a**, Phase-contrast image of a *Xenopus* spinal neuron. G, growth cone; S, soma; N, netrin-1 pipette; Rec, recording pipette. **b**, Sample traces of Ca^{2+} currents in response to voltage steps (-40 to $+40$ mV) in the absence ($-$ netrin-1) or presence ($+$ netrin-1) of a netrin-1 gradient, recorded from the growth cone before (left) and after (centre) addition of nimodipine (20 μM), or from the soma (right). **c**, **d**, Sample traces of Ca^{2+} currents

(inset), summaries of I/V relationship (left) and cumulative distribution of the peak Ca^{2+} currents (right) measured in DCC and DCC–UNC5 neurons. **e**, Average peak amplitude of Ca^{2+} currents in DCC, DCC–UNC5 or NP-1 neurons in the absence or presence of a netrin-1 or Semaphorin 3A gradient. The column labelled 'blank' indicates no netrin-1 gradient. Significance test as in Fig. 1c. Scales: 500 pA, 20 ms.

currents through LCCs in the growth cones of UNC5-overexpressing neurons (Fig. 2d, e). However, application of *Sema3A* had no effect on Ca^{2+} currents in these neurons (Fig. 2e). Notably, bath application of either 8-Br-cGMP or KT5720 reduced Ca^{2+} currents in control neurons either in the presence or absence of netrin-1 (Fig. 2c, e), similar to that found in netrin-1-treated UNC5-overexpressing neurons (Fig. 2d, e). Consistently, bath application of KT5823 significantly rescued Ca^{2+} currents in UNC5-overexpressing neurons (Fig. 2d, e). Thus, a constitutive level of cAMP signalling is required to maintain the basal activity of LCCs in control neurons. Moreover, conditions that trigger attractive or repulsive growth-cone turning were found to induce, respectively, enhanced or reduced Ca^{2+} currents in growth cones. The bidirectional modulation of growth-cone turning by cyclic nucleotide pathways thus directly reflects a modulation of LCC activity in response to netrin-1.

Modulation of ion channels induced by ligand-receptor activation is mediated by second messengers in a variety of neurons¹⁸. Serotonin-induced activation of *Aplysia* sensory neurons is mediated by cAMP signalling and is antagonized by the tetrapeptide FMRFamide through the action of the arachidonate 12-lipoxygenase

metabolite 12-hydroperoxyeicosatetraenoic acid (12-HPETE)¹⁹. This antagonism is due to the converging actions of cAMP and 12-HPETE signalling pathways on the same K^{+} channel¹⁹. Because 12-HPETE regulates the cGMP level by direct activation of soluble guanylate cyclase³, and 12-lipoxygenase is expressed in mammalian neurons^{20,21} and in *Xenopus* oocytes²², we examined the possibility that 12-HPETE is a downstream effector of DCC-UNC5-mediated signalling. Application of exogenous 12-HPETE (500 nM) indeed converted the netrin-1-induced attraction to repulsion in control neurons (Fig. 3a, e), but had no significant effect on repulsion in UNC5-overexpressing neurons (Fig. 3c, e). Furthermore, it significantly reduced Ca^{2+} currents in growth cones of control neurons (Fig. 3b, f). Because a specific 12-lipoxygenase inhibitor, baicalein (10 μM), had no significant effect on netrin-1-induced attraction (Fig. 3a, e), the 12-HPETE-cGMP pathway did not seem to be activated by netrin-1 in control neurons. However, in neurons overexpressing UNC5, baicalein (10 μM) converted netrin-1-induced repulsion to attraction (Fig. 3c, e) and rescued the Ca^{2+} currents (Fig. 3d, f). Although it has been reported that 12-hydroxyeicosatetraenoic acid (12-HETE), a stable by-product of 12-HPETE, is involved in the collapse of rat sensory growth cones

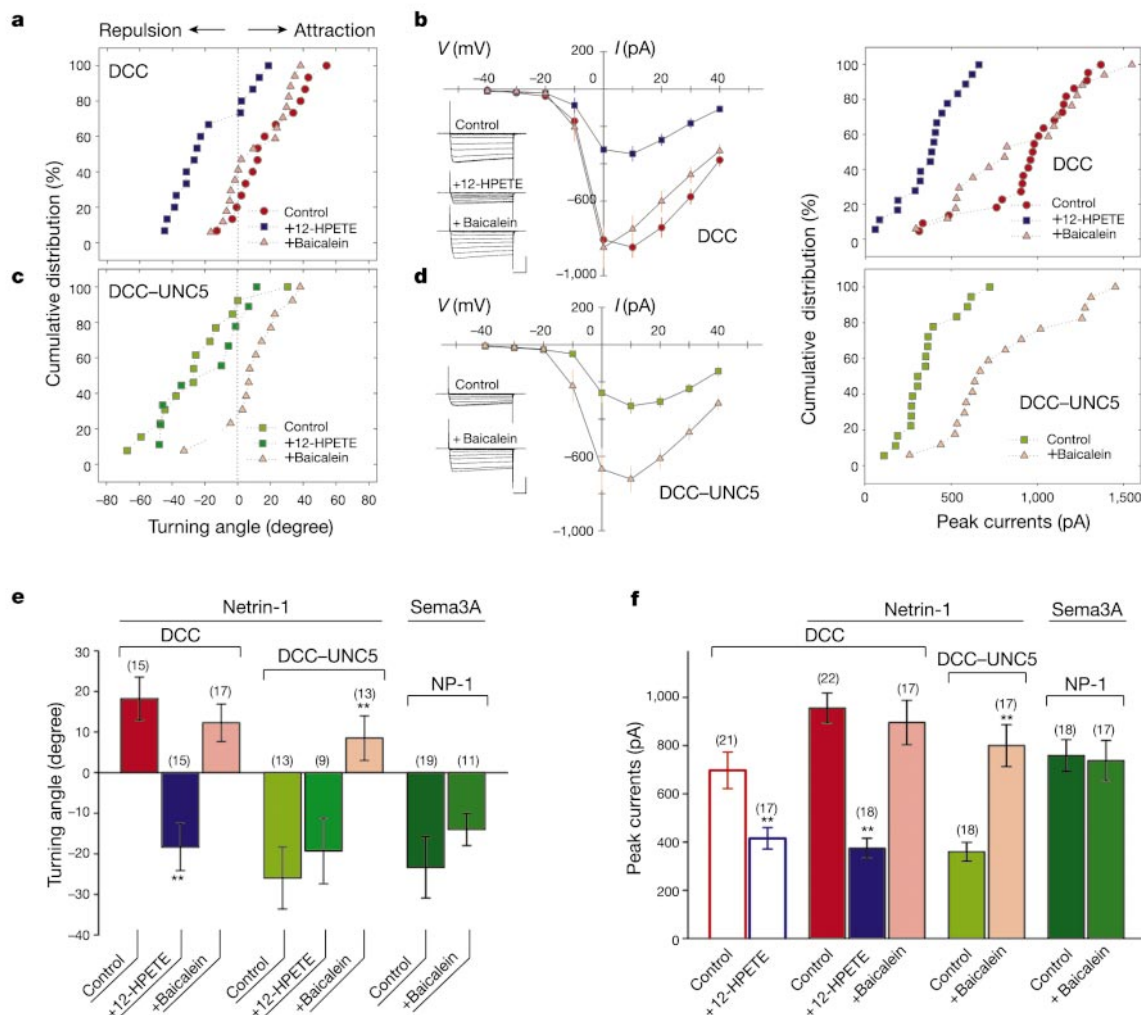


Figure 3 12-HPETE is required for DCC-UNC5-mediated repulsion. **a, c**, Distribution of turning angles in response to a netrin-1 gradient for DCC (**a**) and DCC-UNC5 (**c**) neurons in culture medium alone (control) or after addition of 12-HPETE or baicalein. **b, d**, Ca^{2+} currents in DCC (**b**) and DCC-UNC5 (**d**) neurons under the conditions described in **a, c**.

e, Average turning angles in response to a netrin-1 or *Sema3A* gradient, for DCC, DCC-UNC5 or NP-1 neurons, under various conditions. **f**, Average peak amplitude of Ca^{2+} currents, corresponding to the experimental conditions described in **e**. Significance test as in Fig. 1c. Scales; 500 pA, 20 ms.

induced by *Sema3A* (ref. 23), we found that baicalein treatment had no significant effect on NP-1-mediated repulsion, nor on growth-cone Ca^{2+} currents in the presence of *Sema3A* (Fig. 3e, f). These results suggest that 12-HPETE activates a cGMP-dependent pathway and is a specific downstream effector of DCC-UNC5 signalling, although a role for other metabolites cannot be excluded. These results provide further support for the hypothesis that distinct pathways underlie netrin-1- and *Sema3A*-induced repulsion.

Our findings suggest that modulation of Ca^{2+} channels through cyclic nucleotide-dependent signalling pathways may directly mediate netrin-1-induced bi-directional growth-cone guidance. Activation of DCC and DCC-UNC5 by netrin-1 may trigger two different cyclic nucleotide signalling pathways (Fig. 4). In our model, activation of DCC leads to activation of LCCs². In the absence of UNC5, DCC activation also triggers a cAMP-dependent signalling pathway^{6,24} and enhances LCC activity¹³ (Fig. 4a). Enhanced Ca^{2+} entry, together with additional Ca^{2+} release from RyRs and inositol 1,4,5-trisphosphate receptors in the endoplasmic reticulum (ER)^{25,26}, creates a high-level gradient of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) favouring growth-cone attraction². In the presence of UNC5 (Fig. 4b), netrin-1 activation of DCC-UNC5 also triggers cGMP signalling, mediated by 12-HPETE. Activation of cGMP signalling results in a lower-level $[\text{Ca}^{2+}]_i$ gradient and growth-cone repulsion². Thus, different spatial patterns of $[\text{Ca}^{2+}]_i$ at the growth cone are responsible for activating different sets of downstream effectors resulting in bi-directional turning responses. This notion is further supported by observations

shown in Fig. 1. As shown in Fig. 4c, in DCC-expressing neurons, a netrin-1 gradient was attractive (high $[\text{cAMP}]_i/[\text{cGMP}]_i$ or $[\text{Ca}^{2+}]_i$) either under normal conditions or with PKG inhibition, but became repulsive when PKA activity was inhibited or when cGMP signalling was activated (low $[\text{cAMP}]_i/[\text{cGMP}]_i$ or $[\text{Ca}^{2+}]_i$). Conversely, in neurons overexpressing UNC5, netrin-1-induced activation of DCC-UNC5 leads to increased cGMP signalling (lower $[\text{cAMP}]_i/[\text{cGMP}]_i$ and $[\text{Ca}^{2+}]_i$) resulting in repulsion, which can be converted to attraction by PKG inhibition. Finally, in either DCC- or DCC-UNC5-expressing neurons, simultaneous inhibition of PKA activity (with KT5720) and activation of cGMP signalling (using either 8-Br-cGMP or activation of the DCC-UNC5 complex) resulted in an abrogation of netrin-1-induced turning, presumably because this combination suppresses Ca^{2+} signals altogether.

Our results strongly support the hypothesis that bi-directional turning responses of nerve growth cones to netrin-1 depend on the relative activities of cAMP- and cGMP-dependent pathways, which regulate the cytosolic level of Ca^{2+} signals by modulating Ca^{2+} channels in the plasma membrane and ER^{27} . We also demonstrated a possible role for the lipid 12-HPETE as an effector of UNC5 signalling in triggering repulsive growth-cone turning. Our results show that the interaction of cyclic nucleotide and Ca^{2+} signalling pathways is critical in determining the direction of growth-cone extension, and that regulation of Ca^{2+} channels is a pivotal early event in the transduction of netrin-1 signals. □

Methods

In vitro transcription

Capped messenger RNAs were synthesized using mMESSAGE mMACHINE (Ambion) as described by the manufacturer. The transcription products were purified using the QIAquick PCR Purification Kit (Qiagen). The integrity of mRNAs was examined by agarose gel electrophoresis.

Xenopus microinjection and primary spinal neuron cultures

In vitro fertilization was performed as described²⁷. *Unc5H2* mRNA was injected into two blastomeres of *Xenopus laevis* embryos at the four-cell stage. For each blastomere, we injected 4–8 nl of mRNA solution containing $0.125 \mu\text{g} \mu\text{l}^{-1}$ *Unc5H2* and $0.25 \mu\text{g} \mu\text{l}^{-1}$ green fluorescent protein (GFP) mRNA. GFP was used as an indicator for expression of the sample mRNA. Cultures of spinal neurons were prepared from neural tube tissues of stage 22 (for netrin-1 assays) and 26 (for *Sema3A* assays) *Xenopus* embryos, as described previously²⁴. Cultures were used between 14–20 h after plating at room temperature (20–22 °C). Culture medium consisted of 49% (v/v) Leibovitz medium (GIBCO), 1% (v/v) fetal bovine serum (HyClone) and 50% (v/v) Ringer's solution (in mM: 115 NaCl, 2 CaCl_2 , 2.5 KCl and 10 HEPES, pH 7.4).

Guidance proteins and pharmacological usage

Netrin-1 ($5 \mu\text{g} \text{ml}^{-1}$)²⁴, *Sema3A* protein ($3,000 \text{ U} \text{ml}^{-1}$)²⁸ and 12-HPETE (500 nM)²⁹ were prepared as described. Agonists and antagonists were purchased from Calbiochem. The biological activity of *Sema3A* ($\text{U} \text{ml}^{-1}$) was evaluated using a collapse assay in chick DRG neurons³⁰. All pharmacological agents, except 12-HPETE, were applied in the bath 30 min before experiments. 12-HPETE was applied in the bath 3 min before the turning assay (500 nM) or was used to generate a gradient (0.5 mM in the pipette) through a micropipette using pulsatile ejection during electrophysiological studies. Stability of 12-HPETE in the bath, as tested previously²⁹, was at least 6 min at 37 °C.

Growth-cone turning assay

Microscopic gradients of guidance proteins were produced as previously described²⁴. Microscopic images of neurites were recorded with a CCD (charge-coupled device) camera (Hitachi KP-M2U) attached to a phase contrast microscope (Olympus CK-40) and analysed using NIH Image 1.62. The final turning angle, defined by the angle between the original direction of neurite extension and a straight line connecting the positions of the growth cone at the onset and the end of the 1-h period, was measured. Only those growth cones with net extension $>7 \mu\text{m}$ over the 1-h period were included for analysis.

Electrophysiology

Whole-cell voltage-clamp recordings were made in *Xenopus* spinal neuron growth cones or soma using pipettes with resistances ranging from 5 to $8 \text{ M}\Omega$ or 4 to $6 \text{ M}\Omega$, respectively, using a patch-clamp amplifier (Axopatch 200B, Axon). Recording pipettes were filled with an internal solution of (in mM): 115 CsCl, 10 HEPES, 10 TEA-Cl, 10 EGTA, 4 Mg-ATP, and was adjusted to pH 7.4 with CsOH. The bathing solution contained (in mM): 80 NaCl, 35 TEA-Cl, 10 CaCl_2 , 10 HEPES, 1×10^{-6} TTX, and was adjusted to pH 7.4 with NaOH. A series resistance $\leq 15 \text{ M}\Omega$ was deemed acceptable, and was compensated by 40–50% with a $10\text{-}\mu\text{s}$ lag time. Whole-cell Ca^{2+} currents evoked by step depolarization from a holding

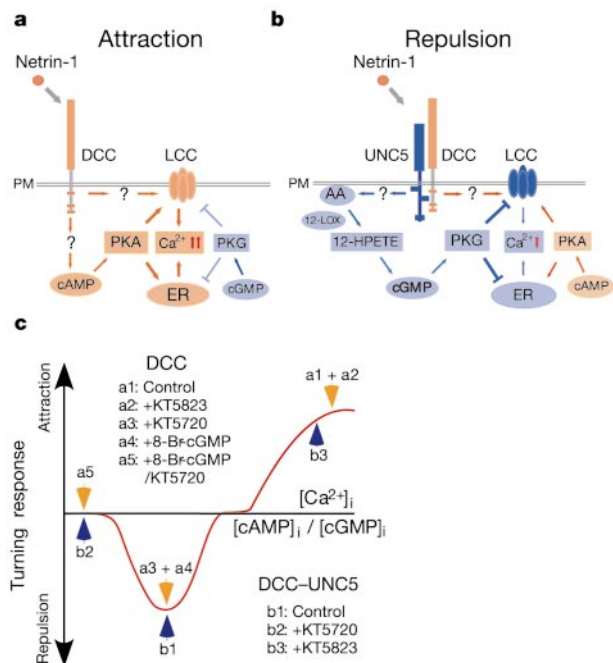


Figure 4 Model for netrin-1-induced second messenger signalling. **a**, For attraction, DCC activation by a netrin-1 gradient creates a high-level $[\text{Ca}^{2+}]_i$ gradient by triggering LCC activity and by stimulating the cAMP–PKA pathway, which further activates LCC in the plasma membrane (PM) and Ca^{2+} channels in the ER. **b**, For repulsion, activation of DCC–UNC5 by a netrin-1 gradient results in a low-level $[\text{Ca}^{2+}]_i$ gradient by triggering LCC activity and the arachidonic acid pathway, which in turn elevates 12-HPETE and cGMP, and consequently inactivates LCC and Ca^{2+} channels in the ER. **c**, Summary of $[\text{cAMP}]_i/[\text{cGMP}]_i$ - and $[\text{Ca}^{2+}]_i$ -dependent growth-cone turning responses to netrin-1.

potential of -40 mV to $+40$ mV (each step 10 mV for 100 ms)¹⁷ were recorded with the patch-clamp amplifier. Data were filtered at 2 kHz and collected at 10 kHz. Ca^{2+} channel activity was evaluated by measuring peak inward currents.

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LAF1 ubiquitination by COP1 controls photomorphogenesis and is stimulated by SPA1

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Far-red light regulates many aspects of seedling development, such as inhibition of hypocotyl elongation and the promotion of greening¹, acting in part through phytochrome A (phyA). The RING motif protein COP1 is also important because *cop1* mutants exhibit constitutive photomorphogenesis in darkness^{2,3}. COP1 is present in the nucleus in darkness but is gradually relocated to the cytoplasm upon illumination⁴. Here we show that COP1 functions as an E3 ligase ubiquitinating both itself and the myb transcription activator LAF1, which is required for complete phyA responses⁵. In transgenic plants, inducible COP1 overexpression leads to a decrease in LAF1 concentrations, but is blocked by the proteasome inhibitor MG132. The coiled-coil domain of SPA1, a negative regulator of phyA signalling⁶, has no effect on COP1 auto-ubiquitination but facilitates LAF1 ubiquitination at low COP1 concentrations. These results indicate that, in darkness, COP1 functions as a repressor of photomorphogenesis by promoting the ubiquitin-mediated proteolysis of a subset of positive regulators, including LAF1. After the activation of phyA, SPA1 stimulates the E3 activity of residual nuclear COP1 to ubiquitinate LAF1, thereby desensitizing phyA signals.

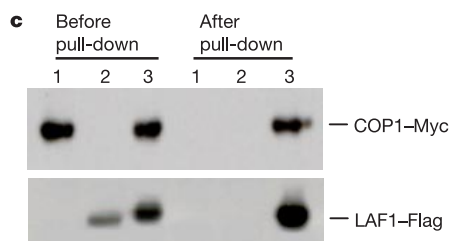
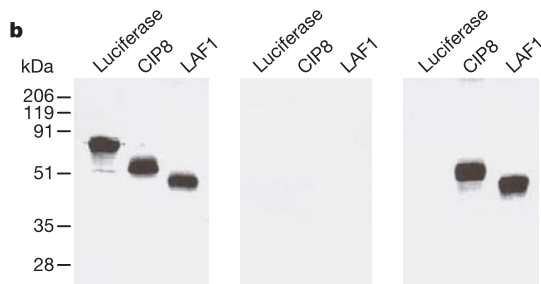
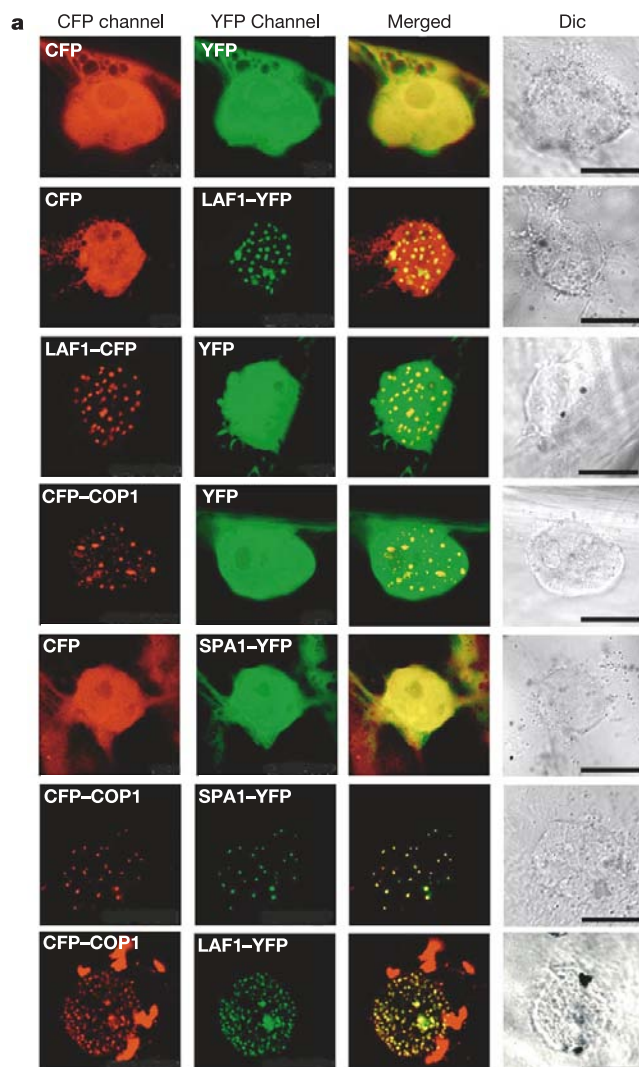
In plants, the light signal is perceived by different photoreceptors that activate a network of signalling intermediates to control the expression of hundreds of genes⁷. The phytochrome photoreceptor family regulates downstream responses by switching between biologically inactive and active forms in response to absorbing red or far-red light, respectively⁸. Several *Arabidopsis* mutants deficient in intermediates of the phyA signalling pathway have been identified. Some of these show reduced responses, whereas others are hypersensitive^{9,10}. The COP/DET/FUS proteins have been proposed to act as negative regulators of photomorphogenic development because their loss-of-function mutants develop as light-grown plants in darkness¹¹. Among these repressors, the RING motif protein COP1 seems to be a key regulator that interacts with components of the phyA signalling pathway such as HY5 (ref. 12) and SPA1 (ref. 13).

LAF 1 is a myb transcription activator that participates in the transmission of phyA signals to downstream responses; this activator is localized in nuclear bodies⁵. Because COP1 is also found in nuclear bodies¹⁴, we asked whether the two proteins are localized together. To this end, we transiently expressed LAF1 tagged with yellow fluorescent protein (YFP) and COP1 tagged with cyan fluorescent protein (CFP) in onion epidermal cells. Figure 1a shows that the two proteins were indeed localized in the same nuclear bodies, although COP1 was also found in cytoplasmic regions not containing LAF1, which is strictly localized in the nucleus.

Similar experiments showed that SPA1 was distributed in the nucleus and the cytosol but it could be relocated by COP1 to the same LAF1/COP1 nuclear bodies, which is consistent with an interaction between SPA1 and COP1 *in vitro*¹³.

The co-localization of LAF1 and COP1 indicates that the two proteins might interact. To study this, we performed a pull-down of products translated *in vitro* by using maltose-binding protein (MBP) and MBP-COP1. Control experiments showed that MBP alone did not interact with any of the products translated *in vitro*. As expected, COP1 interacted with CIP8 (ref. 15) but not luciferase

(Fig. 1b) and, under the same conditions, LAF1 translated *in vitro* purified together with COP1 (Fig. 1b). The interaction between LAF1 and COP1 also occurred *in vivo*, as confirmed by co-purification experiments (Fig. 1c).



Being a RING motif protein, COP1 has been proposed to act as an E3 ubiquitin protein ligase, with its interacting protein, HY5, as its putative substrate¹⁶. Because no E3 activity has been directly associated with COP1, it has not been possible to verify whether the observed effect of COP1 on HY5 stability¹⁶ is direct or indirect. Indeed, recent work showed that HY5 can be ubiquitinated by CIP8, a cytoplasmic RING motif protein, instead of COP1 (ref. 17). Moreover, other than the putative substrate HY5, no substrate of COP1 has yet been identified. To test whether COP1 is indeed an E3 ligase, we produced the protein in *Escherichia coli* and assayed its E3 activity *in vitro*. Figure 2a shows that COP1 was able to catalyse self-ubiquitination in a reaction that required both E1 and E2 activities. The reaction was dependent on Zn²⁺ ions (Fig. 2b) and required an intact RING motif because Cys → Ser substitutions in residues 52 and 55 of COP1 abolished its E3 activity (Fig. 2c). Moreover, wild-type COP1 E3 activity was blocked by a 2–10-fold excess of the COP1 mutant (C52S; C55S), whereas a 10-fold excess of MBP alone had no inhibitory effect (Fig. 2d). These results indicate that COP1 E3 activity might require dimerization and that excess amounts of the mutant (C52S; C55S) can inhibit E3 activity by forming inactive dimers with wild-type COP1, thus acting in a dominant-negative manner. The coiled-coil region (amino acids 110–255) of COP1 has been shown to be involved in its dimerization¹⁸. Our observation that COP1 dimerization activates its E3 activity confirms previous results with another RING protein¹⁹.

The interaction between LAF1 and COP1 prompted us to investigate whether the former might be a substrate for the latter. Indeed, LAF1 was ubiquitinated by COP1 in a reaction that was also dependent on E1 and E2 activities (Fig. 3a). This reaction was specific, because no LAF1 ubiquitination was detected with SINAT5 (Fig. 3b), an *Arabidopsis* E3 ligase that modifies NAC1 (ref. 19). LAF1 ubiquitination was dependent on the RING motif but not the WD40 domain of COP1 (Fig. 3c).

Additional evidence of LAF1 ubiquitination by COP1 was obtained by comparing reaction products with glutathione S transferase (GST)-labelled ubiquitin and hexahistidine (His₆)-labelled ubiquitin. Fig. 3d shows that the mobility difference of ubiquitinated LAF1 products is consistent with the difference in molecular mass between GST-ubiquitin and His₆-ubiquitin.

LAF1–COP1 interaction and the ubiquitination of LAF1 by COP1 predict that LAF1 concentrations might be reduced by COP1 overexpression *in vivo*. We therefore determined LAF1 concentrations in transgenic plants carrying a 35S–LAF1–HA₃ transgene (where HA encodes haemagglutinin) and an oestradiol-inducible²⁰ XVE–COP1–Myc₆ transgene. In non-induced plants without COP1 overexpression, LAF1 concentrations can be increased about 3.5-fold by MG132, indicating possible proteasomal degradation (Fig. 3e). The induced expression of COP1 noticeably decreased LAF1 concentrations; however, these concentrations could be elevated to the same as those in non-induced plants treated with MG132. LAF1 transcript concentrations were comparable under these four conditions. Taken together, these results show that LAF1 stability *in vivo* can be regulated by COP1 through a proteasome-mediated process. Consistent with its self-ubiquitination activity (Fig. 2) was the observation that COP1

Figure 1 Interaction of LAF1, COP1 and SPA1. **a**, LAF1, COP1 and SPA1 localize together to nuclear bodies in onion epidermal cells. Scale bar, 25 μm. DIC, differential interference contrast. **b**, LAF1 binds to COP1 *in vitro*. MBP–COP1 was mixed with *in vitro* translated luciferase (negative control), CIP8 (positive control) and LAF1. Left panel, autoradiogram of SDS gels of proteins translated *in vitro*; middle panel, autoradiogram after binding with MBP resin; right panel, autoradiogram after binding with MBP–COP1. **c**, LAF1 interacts with COP1 *in vivo*. Lane 1, transgenic plant expressing COP1–Myc₆; lane 2, transgenic plant expressing LAF1–Flag₃; lane 3, transgenic plant expressing COP1–Myc₆ and His₆–LAF1–Flag₃.

concentrations were similarly increased by MG132 (Fig. 3e), indicating possible proteasomal regulation as well.

Of the various loci required for phyA signalling, only *EID1* (ref. 21) and *SPA1* (ref. 22) encode negative regulators. Because SPA1 interacts with COP1 through its coiled-coil domain¹³, we speculated that this protein might inhibit COP1 auto-ubiquitination or facilitate LAF1 ubiquitination by COP1, or both. To examine these possibilities, we produced a GST fusion protein containing the coiled-coil domain of SPA1 (GST-SPA1cc). Figure 4a shows that this fusion protein had no significant effect on COP1 auto-ubiquitination over a 40-fold range of COP1 concentrations. By contrast, at 600 ng COP1, increasing amounts of GST-SPA1cc (0–1.2 µg) stimulated LAF1 ubiquitination, whereas GST alone had no noticeable effect (Fig. 4b). In this series of experiments, the stimulatory effect was saturated at 300 ng GST-SPA1cc. We next examined the effect of 300 ng GST-SPA1cc on LAF1 ubiquitination over a range of COP1 concentrations, using GST as a negative control. Whereas GST-SPA1cc had little effect on LAF1 ubiquitination when COP1 was present in large amounts (0.6–1.2 µg; Fig. 4c and data not shown), it had a stimulatory effect that became more pronounced as the amount of COP1 decreased below 600 ng (Fig. 4c and data not shown). Similar results were obtained using His₆-SPA1cc (data not shown). These results show that GST-SPA1cc stimulates LAF1 ubiquitination by COP1 when the latter is present at low concentrations.

The *in vitro* and *in vivo* results described here provide a mechan-

istic explanation of data obtained *in vivo* with *Arabidopsis* mutants and transgenic plants implicated in phyA signalling^{2,6}. Previous work showed that *cop1* mutants display constitutive photomorphogenesis in darkness, indicating that the COP1 protein might act as a repressor of light responses. Here we show that COP1 is an E3 ligase and that it can polyubiquitinate LAF1, a transcription activator, presumably tagging the latter for subsequent proteolysis. This observation is consistent with the notion that, in darkness, COP1 might repress light responses by targeting a subset of positive regulators such as LAF1 for destruction. On exposure to light, depletion of COP1 in the nucleus⁴ allows the positive regulators to accumulate and to promote downstream photomorphogenic responses.

Our results with SPA1cc indicate that, after the activation of phyA, residual nuclear COP1 continues to have a role in regulating phyA responses, but now in concert with SPA1. SPA1 is a nuclear protein synthesized in response to far-red light²². We show here that

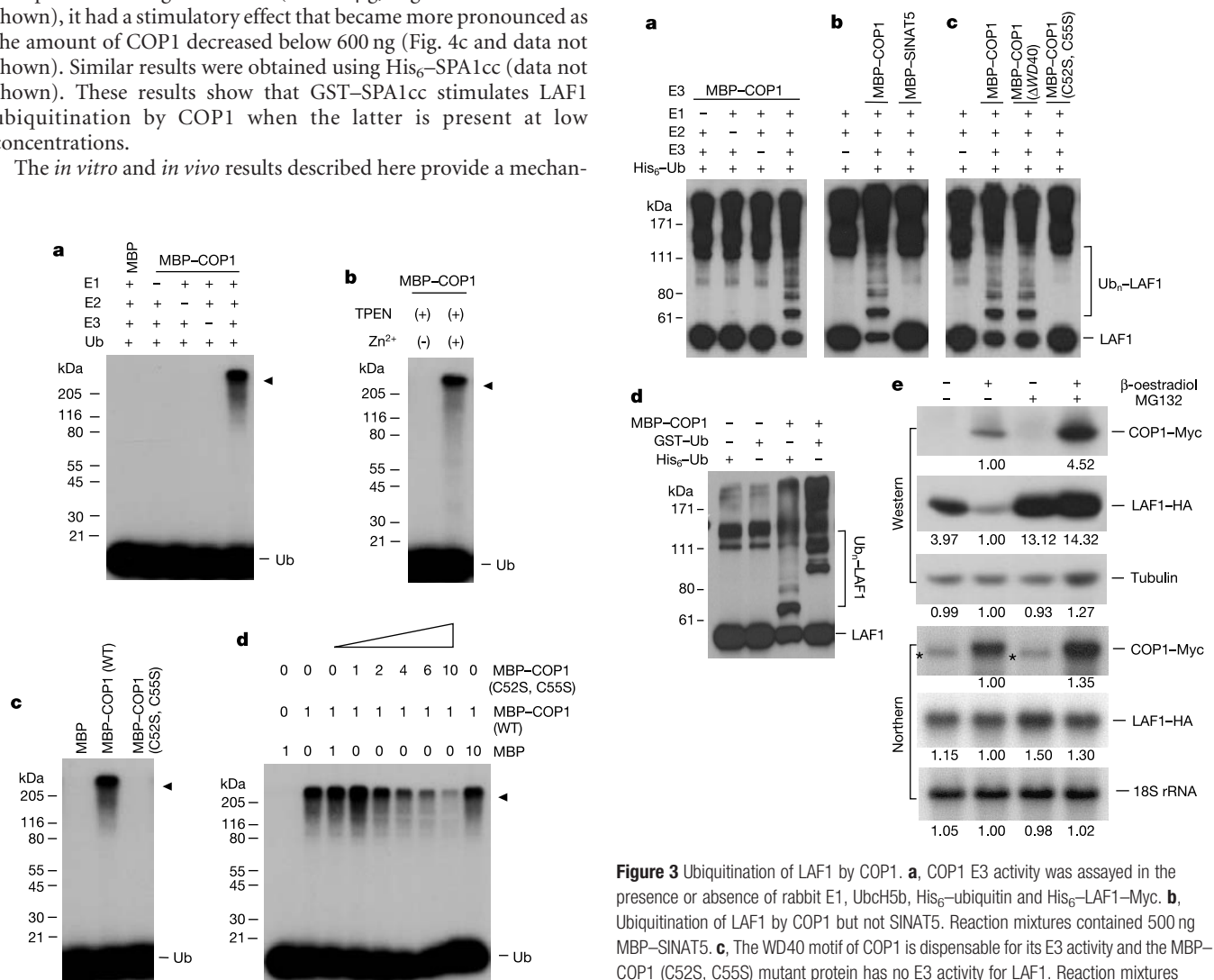


Figure 2 COP1 self-ubiquitination activity. **a**, MBP-COP1 was assayed for E3 activity by using ³²P-labelled ubiquitin (Ub) in the presence or absence of E1 and E2. **b**, COP1 E3 activity requires Zn²⁺. MBP-COP1 was treated with TPEN and reconstituted with (+) or without (-) Zn²⁺. **c**, MBP-COP1 E3 activity is dependent on its RING motif. Wild-type (WT) MBP-COP1 and MBP-COP1 mutant (C52S, C55S) were assayed for self-ubiquitination. **d**, COP1 mutant (C52S, C55S) blocks COP1 E3 activity. Numbers indicate the relative amounts of proteins present in the reaction, where 1 represents 200 ng MBP, MBP-COP1 or MBP-COP1 (C52S, C55S). Arrowheads indicate polyubiquitinated COP1.

Figure 3 Ubiquitination of LAF1 by COP1. **a**, COP1 E3 activity was assayed in the presence or absence of rabbit E1, UbcH5b, His₆-ubiquitin and His₆-LAF1-Myc. **b**, Ubiquitination of LAF1 by COP1 but not SINAT5. Reaction mixtures contained 500 ng MBP-SINAT5. **c**, The WD40 motif of COP1 is dispensable for its E3 activity and the MBP-COP1 (C52S, C55S) mutant protein has no E3 activity for LAF1. Reaction mixtures contained 500 ng COP1 (wild-type, ΔWD40 or C52S, C55S). Ub, ubiquitin. **d**, COP1 E3 activity was assayed with GST-ubiquitin. **e**, Degradation of LAF1 by COP1 *in vivo* is mediated by the proteasome pathway. Double transgenic plants of *35S-LAF1-HA* and *XVE-COP1-Myc* were induced by β-oestradiol and then treated with or without MG132. After incubation overnight, COP1 and LAF1 concentrations were assessed by western blotting with tubulin concentrations as loading controls. RNA concentrations (5 µg per lane) were determined by northern blotting, with 18S rRNA as a loading control. In the top northern panel, asterisks indicate endogenous *cop1* transcript. Numbers under lanes indicate relative intensities.

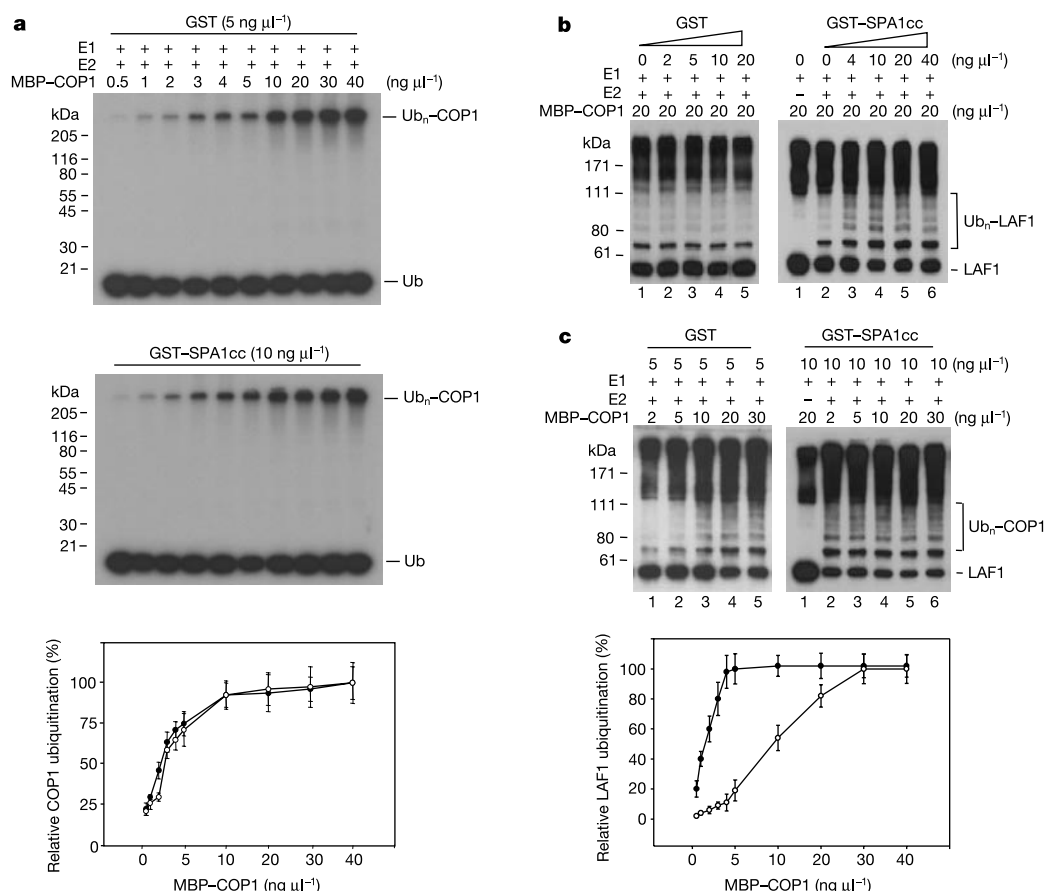


Figure 4 SPA1cc modulates COP1 E3 activity. **a**, Effect of GST-SPA1cc on COP1 self-ubiquitination activity. Reactions were performed with GST-SPA1cc (filled circles) or GST (open circles). The relative concentrations of ubiquitin n -COP1 (Ub n -COP1) were measured by scanning the relevant regions of the X-ray film with the Scion image program. Percentages are relative to the amount of ubiquitin n -COP1 at 1.2 μg MBP-COP1. Results are for $n = 3$ and are means $\pm$ s.e.m. **b**, Effect of SPA1cc on LAF1

ubiquitination at 600 ng COP1. **c**, Effect of COP1 on LAF1 ubiquitination at 300 ng SPA1cc. Reactions were performed with GST-SPA1cc (filled circles) or GST (open circles). The relative concentrations of ubiquitin n -LAF1 were measured by scanning regions of the X-ray film corresponding to the 61–111-kDa range with the Scion image program. Percentages are relative to the amount of ubiquitin n -LAF1 at 1.2 μg MBP-COP1. Results are for $n = 5$ and are means $\pm$ s.e.m.

SPA1cc has no significant effect on COP1 auto-ubiquitination activity over a wide range of concentrations, but it stimulates LAF1 ubiquitination at low, but not high, concentrations of the COP1 E3 ligase. These results indicate that at low nuclear COP1 concentrations, which is true shortly after phyA activation, newly synthesized SPA1 might facilitate the ubiquitination of LAF1 by COP1, and probably other positive signalling components, to desensitize the light signal. In *spa1* mutants, the absence of SPA1 is expected to impair COP1 E3 activity severely, resulting in higher concentrations of positive regulators for a given far-red light intensity, and hence the hypersensitive response⁶. It is also possible that in plants grown under light-dark conditions SPA1 might synergize with COP1 in the dark period to deplete transcription activators, like LAF1, more rapidly to prepare plants for the light signals of the following day.

RING motif E3 ligases are known to catalyse both auto-ubiquitination and substrate ubiquitination. How these two opposing reactions are regulated in the cell during signalling is unclear and relatively unexplored. Several regulators have been shown to aid the auto-ubiquitination of DIAP1, a *Drosophila* E3 ligase that inhibits apoptosis^{23–26}, but their effects on substrate ubiquitination are unknown. By contrast, SPA1 has little effect on COP1 auto-ubiquitination but stimulates its E3 activity towards substrates. The mechanism of this stimulation is not yet clear but it does not involve the interaction of SPA1 with the coiled-coil domain of COP1 monomer, because this would dissociate COP1 dimers and

inhibit E3 activity. One possibility is that SPA1 enhances the binding of LAF1 to COP1 at low concentrations of the latter. Subcellular localization experiments demonstrated that in darkness, LAF1 and COP1 are found in nuclear bodies^{5,14} which also contained SPA1. On the basis of the ability of COP1 to ubiquitinate LAF1, these nuclear bodies might be sites of protein degradation similar to the clastosomes of animal cells²⁷. The characterization of additional components of plant clastosomes and the identification of other substrates and regulators of COP1 will lead to a better understanding of the role of regulated proteolysis in phyA signalling. □

Methods

Construction of expression vectors and preparation of recombinant proteins

Sequences encoding full-length COP1 and deletion mutants (WD40, amino acids 1–255; RING, amino acids 110–675; RING/coiled-coil, amino acids 216–675) were amplified by polymerase chain reaction (PCR) and cloned into pMal-c2 (New England Biolabs) to generate complementary DNA molecules encoding fusions with MBP. Mutations in the RING motif (C52S, C55S) were generated with the Quick Change site-directed mutagenesis kit (Promega). Sequences for a carboxy-terminal Myc tag were appended to the full-length LAF1 cDNA by PCR and cloned into pET-28a (+) (Novagen) to generate the coding sequence for His₆-LAF1-Myc. The sequence encoding the coiled-coil domain of SPA1 (SPA1cc; amino acids 521–696) was amplified by PCR and cloned into pGEX4T-1 (Pharmacia).

All recombinant proteins were expressed in *E. coli* strain BL21. For purification of MBP-COP1, bacteria were lysed in 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 1% Triton X-100, 5 mM dithiothreitol (DTT), 2 mM phenylmethylsulphonyl fluoride (PMSF) and a protease inhibitor cocktail (Promega) and purified on amylose resins (New England Biolabs). For His₆-LAF1-Myc purification, bacteria were lysed in 50 mM NaH₂PO₄ pH 8.0, 300 mM NaCl, 1% Triton X-100, 1 mM imidazole, 5 mM DTT, 2 mM PMSF and a

proteinase inhibitor cocktail, and purified on Ni²⁺-nitrilotriacetate (Ni²⁺-NTA) resins (Qiagen). For GST–SPA1 cc purification, bacteria were lysed in PBS pH 7.5 containing 1% Triton X-100, 2 mM PMSF and a proteinase inhibitor cocktail, and purified on glutathione resins (Pharmacia). Protein concentrations were determined by the Bradford assay (Bio-Rad).

Subcellular localization of LAF1, COP1 and SPA1

The CFP and YFP coding sequences were fused in-frame to the 5' end of COP1 cDNA and to the 3' end of SPA1 cDNA, respectively, and cDNA sequences for LAF1–YFP and LAF1–CFP were constructed similarly. All fusion genes or genes for YFP and CFP were expressed from a 35S promoter. Onion epidermal cells were bombarded with different combinations of plasmids with a helium biolistic gun²⁸ and analysed by confocal microscopy after incubation in the dark for 12 h.

In vitro and in vivo binding assays

T7-promoter-driven *in vitro* transcription/translation templates were derived from full-length LAF1 and CIP8 coding sequences^{5,15} cloned into pRSETA (Invitrogen). [³⁵S]methionine-labelled luciferase, LAF1 and CIP8 were generated by transcription and translation *in vitro* with wheatgerm extracts by using a T7/T3 coupled TnT kit (Promega). For binding *in vitro*, 10 µl of the translation mixture was added to 500 µl binding buffer (50 mM HEPES pH 7.5, 1 mM EDTA, 150 mM NaCl, 10% glycerol, 0.1% Tween 20, 0.5 mM DTT) and the mixture was incubated at 25 °C for 1 h. The reaction mixture was incubated with amylose resin beads, which were then washed five times with washing buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.2% Nonidet P40). *In vitro* translation products and COP1-interacting proteins were analysed by SDS–polyacrylamide-gel electrophoresis (SDS–PAGE).

For binding *in vivo*, LAF1–Flag₃ and His₆–LAF1–Flag₃ cDNAs were cloned into pB1121 (Clontech) and COP1–Myc₆ cDNA was cloned into pBA002 (ref. 29). Constructs were transformed into *Arabidopsis thaliana* (Landsberg *erecta*) by vacuum infiltration³⁰. Five-day-old light-grown plants (16 h light/8 h dark) on MS medium were ground in liquid nitrogen and resuspended in an extraction buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1 mM DTT) containing 1 × complete protease inhibitor cocktail (Roche) and 1 mM imidazole (Sigma). After centrifugation, supernatants were purified on Ni²⁺-NTA columns and eluted proteins were detected by western blotting with anti-Myc (Santa Cruz Biotechnology) or anti-Flag (Sigma) antibodies.

In vitro ubiquitination assay

³²P-labelled ubiquitin was prepared with recombinant GST–ubiquitin²⁰. Reaction mixtures (30 µl) contained 500 ng COP1 (E3), 20 ng each of rabbit E1 and UbcH5B (both from Affinity), and either 2 × 10⁴ c.p.m. of ³²P-labelled ubiquitin or 10 µg unlabelled His₆–ubiquitin (Sigma) or GST–ubiquitin (Boston Biochemical) in a buffer containing 50 mM Tris pH 7.4, 2 mM ATP, 5 mM MgCl₂ and 2 mM DTT. After incubation at 30 °C for 2 h, reaction mixtures were heated to 95 °C in SDS–PAGE sample buffer containing 2-mercaptoethanol, before electrophoresis on 8% polyacrylamide SDS gels.

For cation chelation, MBP–COP1 was immobilized on amylose resin and incubated overnight with 2 mM N,N,N',N'-tetrakis(2-pyridylmethyl)-ethylenediamine (TPEN; Biotium) at 4 °C. After extensive washing, the immobilized MBP–COP1 was treated with 20 µM ZnCl₂ for 45 min at 22 °C before the ubiquitination assay.

For LAF1 ubiquitination, each assay contained 10 ng purified His₆–LAF1–Myc. Ubiquitinated LAF1 was detected by western blotting with anti-Myc antibody (Santa Cruz Biotechnology).

Effects of COP1 overexpression on LAF1 concentrations in vivo

Four-day-old light-grown (16 h light/8 h dark) plants carrying 35S–LAF1–HA₃ and XVE–COP1–Myc₆ transgenes on MS medium were treated in the light with or without β-oestradiol for 15 h and then with or without 50 µM MG132 (Calbiochem) for 15 h. Samples were ground in liquid nitrogen and equivalent amounts were analysed by western blotting with anti-HA or anti-Myc antibodies. Plant samples were also processed for northern blotting.

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Enhanced gravi- and phototropism in plant *mdr* mutants mislocalizing the auxin efflux protein PIN1

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Many aspects of plant growth and development are dependent on the flow of the hormone auxin down the plant from the growing shoot tip where it is synthesized^{1,2}. The direction of auxin transport in stems is believed to result from the basal localization within cells of the PIN1 membrane protein, which controls the efflux of the auxin anion³. Mutations in two genes homologous to those encoding the P-glycoprotein ABC transporters that are

especially abundant in multidrug-resistant tumour cells in animals⁴ were recently shown to block polar auxin transport in the hypocotyls of *Arabidopsis* seedlings⁵. Here we show that the *mdr* mutants display faster and greater gravitropism and enhanced phototropism instead of the impaired curvature development expected in mutants lacking polar auxin transport. We find that these phenotypes result from a disruption of the normal accumulation of PIN1 protein along the basal end of hypocotyl cells associated with basipetal auxin flow. Lateral auxin conductance becomes relatively larger as a result, enhancing the growth differentials responsible for tropic responses.

Twenty of the more than 100 genes encoding ABC transporters in the genome of *Arabidopsis* are homologous with the well-studied mammalian multidrug resistance (*MDR*) genes that encode the P-glycoproteins (PGPs)⁶. A role in auxin biology for *Arabidopsis* *MDR*-like proteins was indicated by the finding that the auxin transport inhibitor (1-naphthylphthalamic acid, NPA) bound tightly and specifically to certain members of the group and that mutation of two related *MDR*-like genes (*MDR1* and *PGP1*) resulted in severely reduced polar auxin transport in seedling hypocotyls and inflorescence stems of mature plants^{5,7}. The phenotypes of null *mdr1* and *mdr1 pgp1* double mutants obtained from T-DNA insertion populations⁵, as well as those of *PGP1* antisense transformants⁸, could be understood as consequences of impaired basipetal auxin flow. No mechanistic explanation of how the *MDR*/*PGP* membrane proteins modulate polar auxin transport was offered at the time, although direct transport of auxin by *MDR* proteins or positive regulation of a PIN-type auxin efflux channel was suggested. A subsequent report showing that NPA blocked the relocalization of PIN1 protein to basal plasma membranes after it had first been delocalized by brefeldin A treatment⁹ suggested to us an entirely different mechanism. Perhaps *MDR* proteins, at least three of which bind NPA^{5,7}, function in the mechanism that concentrates PIN1 to the basal end of stem cells.

A previous study used an antibody raised against the PIN1 membrane protein to demonstrate its accumulation at the basal ends of vascular tissue cells, a finding that was considered strong evidence of a role for PIN1 in directing auxin efflux¹⁰. We used the same antibody in the present study to test the hypothesis that improper localization of PIN1 contributed to the auxin-transport defect in the hypocotyls of *mdr* mutants. Figure 1 shows the results obtained when fixed, immunostained hypocotyls of 5-day-old seedlings were examined with epifluorescence or confocal microscopy (upper and lower panels, respectively). In wild-type seedlings, the antibody showed PIN1 to be basally localized in the living cells of the vascular tissues and the two adjacent cell files (Fig. 1a, e, i, m). Very different results were obtained when PIN1 distribution was imaged in *mdr1* hypocotyls: the *mdr1* mutation perturbed PIN1 localization in all cell types of the hypocotyl, replacing the special basal concentration characteristic of the wild type with diffuse and punctate patterns (Fig. 1c, g). The disruption of localization was less severe in non-vascular tissues of light-grown seedlings (Fig. 1k, o), perhaps because *MDR1* expression is decreased by light⁵. Another family member might partly replace *MDR1* function in this condition. The *pgp1* mutation disrupted PIN1 basal localization to a lesser extent than the *mdr1* mutation, particularly in vascular tissues. The instances of basal localization in upper hypocotyls, similar to that in the wild type (Fig. 1b, j), might explain why the *pgp1* mutation did not impair polar auxin transport or produce the same epinastic cotyledon phenotype as the *mdr1* mutation⁵; more uniform localization of PIN1 was observed in the lower hypocotyls (Fig. 1f, n). Another consistently observed effect of the *pgp1* mutation was a generalized increase in background PIN1 signal.

Combining the *mdr1* and *pgp1* mutations had a strong effect on PIN1 localization. In upper hypocotyls, where cells expand rapidly, the PIN1 signal was continuous around cell membranes in etiolated

seedlings, but was discontinuous and diffuse in light-grown seedlings (Fig. 1d, l). Localization in vascular tissues was completely disrupted in all cases. The waviness of the double-mutant hypocotyl (described more fully below) made it difficult to obtain confocal optical sections that showed the perimeters of entire cells in focus, so the epifluorescence images in Fig. 1d might best represent the PIN1-localization phenotype of the double mutant. The PIN1 mislocalization phenotype of the double mutant was again greater in etiolated seedlings than in light-grown seedlings, perhaps because

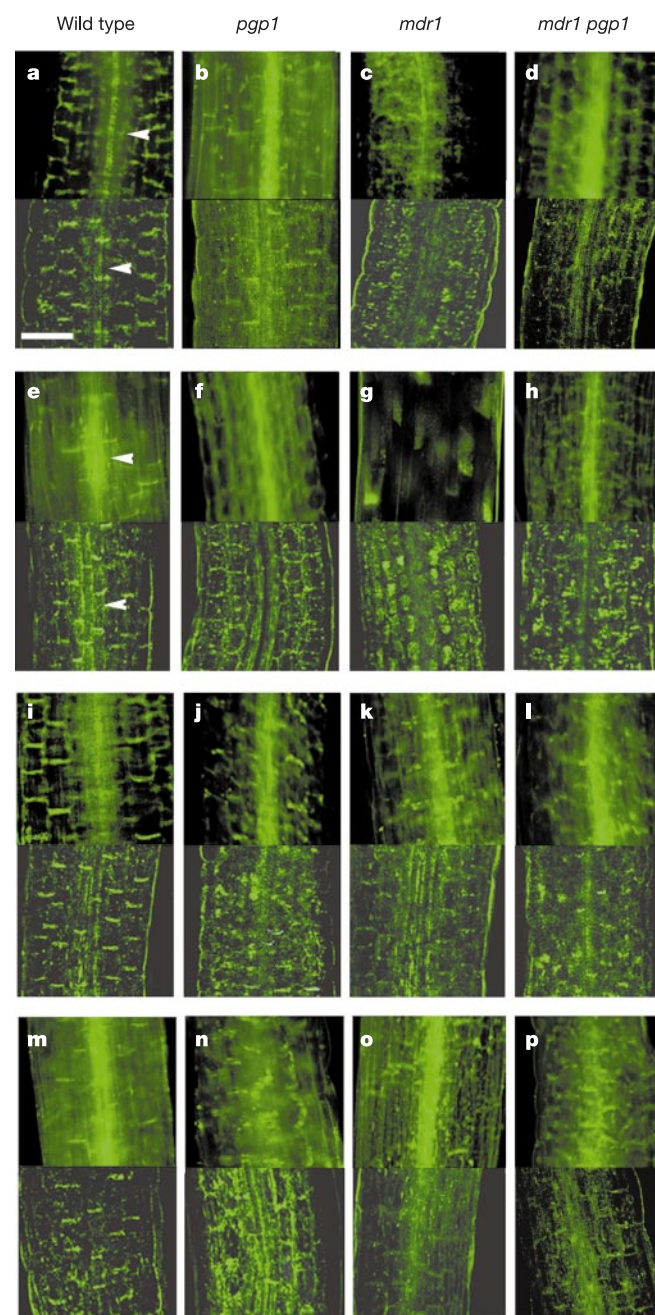


Figure 1 Localization of PIN1 in the hypocotyls of 5-day-old (4 days after germination) wild-type and mutant *Arabidopsis* seedlings. **a–d**, Dark-grown upper hypocotyls. **e–h**, Dark-grown lower hypocotyls. **i–l**, Light-grown upper hypocotyls. **m–p**, Light-grown lower hypocotyls. In each figure part the top panel was photographed by epifluorescence and the bottom panel is a confocal optical section through or near the hypocotyl midplane. Scale bar, 100 μ m. The arrows in **a** and **e** point to the vascular tissue. The localization of PIN1 in older hypocotyls and stems is restricted to vascular tissue. The bright outline in the confocal images is believed to be an optical artefact not representing PIN1 protein.

of the aforementioned effect of light on *MDR1* expression. In the lower portion of light-grown *mdr1 pgp1* hypocotyls, PIN1 staining showed discontinuous, punctate and diffuse basal patterns (Fig. 1h, p). General targeting of proteins to the plasma membrane

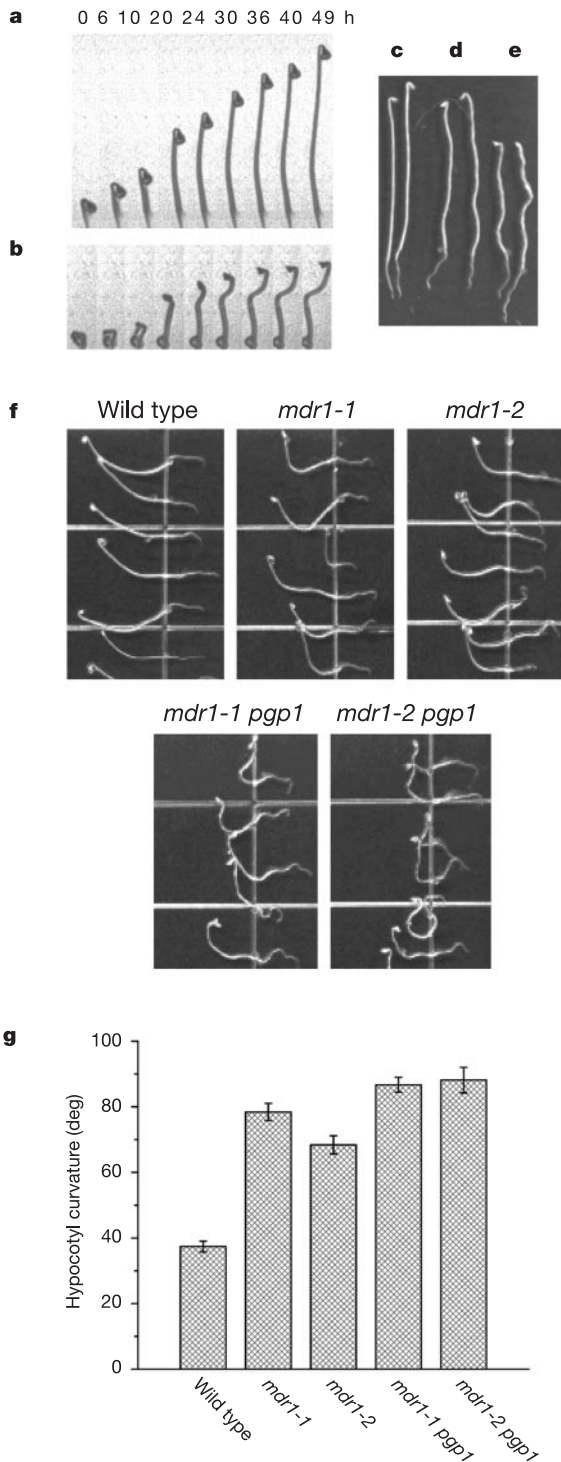


Figure 2 Greater hypocotyl nutation and gravitropism in *mdr* seedlings. **a**, Time course showing mostly straight elongation of a dark-grown wild-type seedling. **b**, Time course showing spurious curvature development during elongation of a *mdr1 pgp1* seedling. **c**, Four-day etiolated wild-type seedlings with characteristically straight hypocotyls. **d**, *mdr1-1* and *mdr1-2* seedlings showing slightly wavy hypocotyls. **e**, *mdr1-1 pgp1* and *mdr1-2 pgp1* seedlings showing shorter, wavier hypocotyls. **f**, Gravitropic angle 18 h after reorientation of dark-grown seedlings through 90°. **g**, Quantification of the final angle achieved by wild-type ($n = 46$) and mutant ($n = 14$ – 25) hypocotyls.

was not perturbed by either mutation alone or in the double mutant because the AHA2 H⁺-ATPase control protein was similarly distributed in all mutants and in the wild type (see Supplementary Figure).

The more generalized distribution of PIN1 in cells of the upper *mdr1 pgp1* hypocotyl, compared with that in the wild type, could be expected to randomize the direction of auxin efflux and thus explain the severe defect in polar auxin transport measured in previous assays⁵. Extra lateral conductance of auxin and diminished basal conductance could also be responsible for the exaggerated curvatures that developed spuriously in the double-mutant hypocotyl as it elongated in darkness (Fig. 2a) compared with the wild type (Fig. 2b).

These stem curvatures, slight in *mdr1* (Fig. 2d) compared with wild-type (Fig. 2c), undetectable in *pgp1* (not shown) but extreme in *mdr1 pgp1* seedlings (Fig. 2e), are due to transient differences in the rate of cell expansion across the stem. Such growth differentials, generally associated with radial gradients of auxin, underlie tropic curvatures induced by stimuli such as asymmetric irradiation or reorientation with respect to the gravity vector. This raised a question about whether tropisms in the mutants would be enhanced, similarly to nutations, or whether they would be impaired, as has been found for other mutants with defects in polar auxin transport¹¹. This question was tested by performing tropism assays: the results were unequivocal (Fig. 2f, g). Etiolated hypocotyls of two different *mdr1* alleles achieved a nearly complete reorientation several hours after being rotated by 90°, whereas the average wild-type response was only half as large. The *pgp1* mutation by itself did not have an apparent effect on gravitropism (data not shown). Quantifying the double-mutant response was more difficult because of enhanced nutation, but it was at least as large as the *mdr1* response.

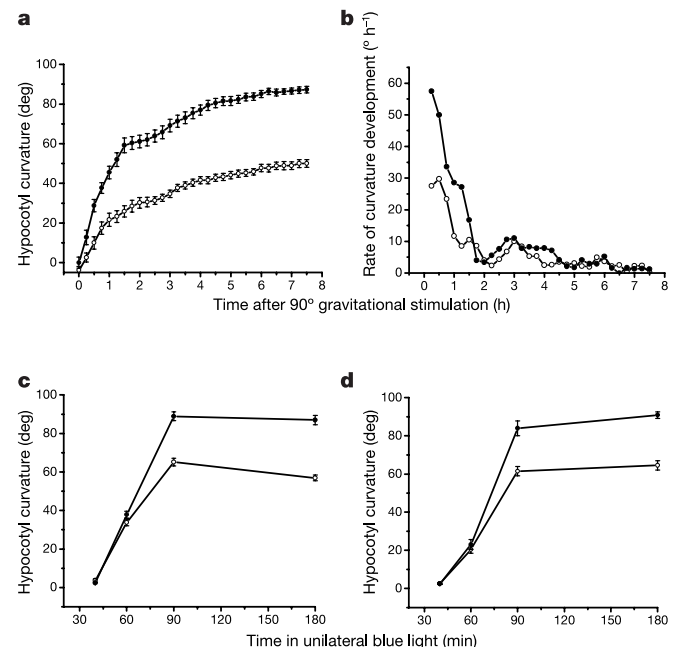


Figure 3 Kinetics of hypocotyl gravitropism and phototropism. **a**, Time series of hypocotyl angle measurements showing that gravitropic curvature developed faster in *mdr1* seedlings (filled circles; $n = 16$) than in wild-type seedlings (open circles; $n = 16$). **b**, The first derivative of the data in **a**, showing that the *mdr1* mutation doubled the rate of curvature development without changing the general time course of the response. Thus, the greater gravitropic angle resulted from a larger difference in growth rate across the stem rather than a similar but more prolonged growth differential. **c**, The *mdr1-1* mutation also increased phototropic curvature development. Etiolated seedlings were irradiated unilaterally with continuous blue light ($n = 17$ for both genotypes). **d**, A second allele of the mutation (*mdr1-2*) displayed similarly greater phototropism than the wild type.

The greater curvature in *mdr1* seedlings could have resulted from a longer period of differential growth or from a steeper growth differential being in effect for a similar period of time. A kinetic analysis of curvature development based on electronic image analysis showed that the latter was true. The hypocotyl angle of wild-type and *mdr1* seedlings was monitored at 15-min intervals after gravitational stimulation (Fig. 3a). A plot of the first derivative of the time series shows the rate of curvature development over time (Fig. 3b) and establishes that *mdr1* hypocotyls curved approximately twice as fast but over the same time course as those of the wild type. Thus, the greater curvature of the *mdr1* hypocotyl was due to a steeper growth differential across the stem, not a more persistent one with the same magnitude as that of the wild type. It would follow, according to the Cholodny–Went theory of tropisms¹¹, that a larger transverse auxin gradient developed in the mutant during this 2-h period. Mislocalized PIN1, together with PIN3 in its normal position along lateral walls¹², could enable greater radial auxin flux, leading to the steeper, although transient, auxin concentration gradient inferred from the kinetics of gravitropic curvature development.

Phototropism, another curvature response driven by a transverse auxin concentration gradient^{11,12}, was about 30% greater in *mdr1* seedlings than in the wild type after 90 min of unilateral blue light. Figure 3c shows data obtained with the *mdr1-1* allele, and Fig. 3d shows that *mdr1-2* seedlings responded very similarly. The enhancement of both gravitropism and phototropism supports the conclusion that MDR1 functions in the growth-control phase of tropisms, as opposed to the stimulus-sensing phase. The initial phase of the phototropic response was not quickened by the *mdr1* mutation to the same extent as the gravitropic response, possibly because MDR1 expression is decreased by the phototropic light stimulus or because the PIN1 localization phenotype was less severe in light-grown *mdr1* hypocotyls (Fig. 1). Future studies will examine whether other MDR family members also influence the distribution of PIN1, whether the interaction is direct or is a result of changes in polarity maintained by auxin gradients, and whether there are specific relationships between particular members of the MDR and PIN families of membrane transport proteins. □

Methods

Plant material

The wild type used in all experiments was the WS ecotype of *Arabidopsis thaliana*. MDR1 and PGPI refer to the loci identified as At3g28860 and At2g36910, respectively. Images were captured with a SPOT camera and processed using Adobe Photoshop 5.0.

Immunolocalization

Arabidopsis seedlings were grown without sucrose on 1% phytar plates, 1/4 Murashige and Skoog basal salts, pH 4.85, at 22 °C, under illumination ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 14 h a day for light-grown seedlings or in complete darkness. Five days after planting, seedlings were prepared for immunolocalization as described previously¹³ with the following changes: seedlings were digested for 90 min in 0.5% pectolyase, 20% Triton X-100; Triton X-100 was omitted from the washes. Affinity-purified anti-PIN1 antibody¹⁰ was diluted 1:400. Affinity-purified anti-AHA2 antibody was diluted 1:300. The secondary antibody was Alexa Fluor 488 conjugate (Molecular Probes, Eugene, Oregon) diluted 1:400. Immunofluorescence imaging was performed with an epifluorescence microscope (Eclipse E 800; Nikon, Melville, New York) equipped with a 450–490-nm excitation filter (model number 41017, Chroma Technology Corporation, Brattleboro, Vermont), a 495-nm dichroic mirror and a 500–530-nm emission barrier pass filter (Chroma), or with a confocal laser scanning microscope (Eclipse 800; Nikon) equipped with an argon laser (488 nm) (Bio-Rad). Green HeNe laser (543 nm, 1.4 mW) and a red laser diode (638 nm, 5 mW) were used for autofluorescence detection. Images were captured with a SPOT camera and processed using Adobe Photoshop 5.0.

Quantifying tropisms

All mutants and double mutants were isolated and constructed as described previously⁵. Seedlings were grown on minimal salt medium (1 mM CaCl_2 , 1 mM KCl, 1% agar). To measure hypocotyl gravitropism, seedlings were grown along the surface of vertical agar plates in light for 1 day, then transferred to darkness for 1.5 days before the plates were reoriented by 90°. The angle made by the hypocotyl apex relative to the original gravity vector was determined from digital images acquired at 15-min intervals after reorientation for the time-course description or after 18 h of gravitational stimulation to determine the final steady-state angle. For phototropism, the seedlings were allowed to grow in darkness

for 2 h before the vertical plate was illuminated from the side with blue light ($0.07 \mu\text{mol m}^{-2} \text{s}^{-1}$) produced by a bank of light-emitting diodes (described previously¹⁴) and passed through a slit before reaching the seedlings. Images of seedlings were recorded, every 10 min after the onset of unilateral irradiation, with a previously described imaging apparatus¹⁴. The angle of curvature was measured from the digital images at selected time points after the response became detectable.

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Chromosome cohesion is regulated by a clock gene paralogue TIM-1

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Faithful transmission of the genome requires that a protein complex called cohesin establishes and maintains the regulated linkage between replicated chromosomes before their segregation^{1,2}. Here we report the unforeseen participation of *Caenorhabditis elegans* TIM-1, a paralogue of the *Drosophila* clock protein TIMELESS, in the regulation of chromosome cohesion. Our biochemical experiments defined the *C. elegans* cohesin complex and revealed its physical association with TIM-1. Functional relevance of the interaction was demonstrated by aberrant

mitotic chromosome behaviour, embryonic lethality and defective meiotic chromosome cohesion caused by the disruption of either TIM-1 or cohesin. TIM-1 depletion prevented the assembly of non-SMC (structural maintenance of chromosome) cohesin subunits onto meiotic chromosomes; however, unexpectedly, a partial cohesin complex composed of SMC components still loaded. Further disruption of cohesin activity in meiosis by the simultaneous depletion of TIM-1 and an SMC subunit decreased homologous chromosome pairing before synapsis, revealing a new role for cohesin in metazoans. On the basis of comparisons between TIMELESS homologues in worms, flies and mice, we propose that chromosome cohesion, rather than circadian clock regulation, is the ancient and conserved function for TIMELESS-like proteins.

Cohesin contains two SMC subunits, Smc1 and Smc3, and two non-SMC subunits, Scc1 (Mcd1/Rad21) and Scc3 (stromal antigen/Psc3)^{1,2}. Cohesin is loaded onto chromosomes before S phase and establishes cohesion between the duplicated chromosomes (sister chromatids) during DNA replication^{1,2}. This regulated linkage is released in preparation for chromosome segregation through a well-defined mechanism that involves the phosphorylation and proteolytic cleavage of the non-SMC cohesin subunit Scc1 (refs 3, 4). By contrast, the mechanisms that underlie the loading and assembly of cohesin onto chromosomes are poorly understood.

To analyse the regulation of chromosome cohesion in *C. elegans*, we first identified homologues of conserved cohesin subunits.

Single candidates for Smc1 (open reading frame (ORF) F28B3.7), Smc3 (ORF Y47D3A.26) and Scc3 (ORF F18E2.3) were found in the *C. elegans* genome database. We showed that *him-1* (high incidence of males)⁵ encodes SMC-1 (see Methods), and *him-1* mutations cause chromosome segregation defects expected for a mutation in a cohesin subunit (see below and ref. 6). Of three candidates⁷ with similarity to cohesin subunit Scc1/Mcd1, COH-2 (ORF F10G7.4) is the true orthologue (see below), prompting us to rename it SCC-1.

True cohesin components in *C. elegans* should assemble into a complex, like those of other organisms. Anti-SMC-1 antibodies precipitated SMC-3, SCC-3 and SCC-1 from embryonic extracts (Fig. 1a). Three controls demonstrated specificity for the co-immunoprecipitation. First, western blots of a mock immunoprecipitation control detected none of the proteins (Fig. 1a). Second, SMC-1 antibodies failed to precipitate two X-chromosome-bound SMC dosage compensation proteins, DPY-27 and MIX-1 (refs 8, 9; Fig. 1a). Last, antibodies against MIX-1, also a condensin complex subunit that assembles onto mitotic chromosomes, failed to precipitate cohesin (Fig. 1a). Reciprocal co-immunoprecipitation of SMC-1, SMC-3 and SCC-3 with anti-SCC-1 antibodies confirmed the formation of a stable cohesin complex (Fig. 1b).

Using a biochemical approach, we sought other components essential for chromosome cohesion. Unique protein bands from SMC-1 immunoprecipitations were identified (Fig. 1c) and analysed by mass spectrometry (Supplementary Table 1). As expected, SMC-1 and SMC-3 were among the proteins in the bands at relative

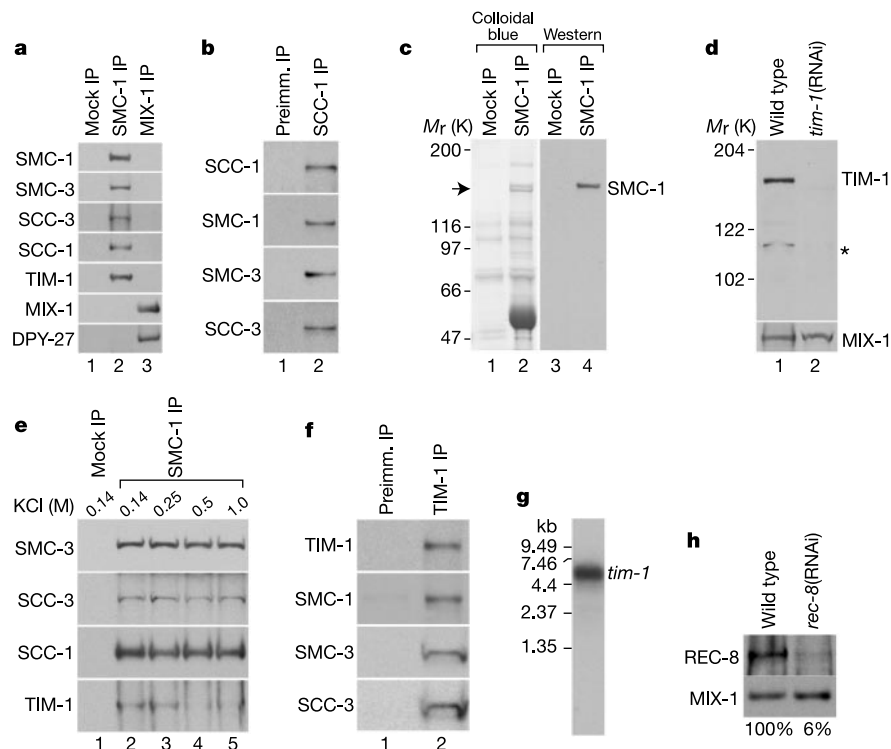


Figure 1 *C. elegans* TIM-1, a paralogue of the circadian clock protein TIMELESS, associates with the cohesin complex. **a–f, h**, Western blots. **a, b, e**, Immunoprecipitation (IP) reactions with antibodies against SMC-1 (**a, e**) or SCC-1 (**b**) co-precipitated the four cohesin subunits from embryonic extracts, indicating the formation of a stable complex. **c**, Cohesin-associated protein bands (arrow), similar in mobility to SMC-1 (M_r 150K), were identified by comparison of mock and SMC-1 immunoprecipitations stained with colloidal blue. Peptide mass fingerprinting showed that the bands included SMC-1, SMC-3 and TIM-1 (Supplementary Table 1). The M_r 180K band includes vitellogenin, a common contaminant in embryonic extracts. **d**, Antibodies against TIM-1 specifically

recognized full-length (M_r 150K) TIM-1 and a post-lysis cleavage product of M_r 120K (asterisk) from whole worm lysates; both proteins, but not MIX-1, were depleted by *tim-1* RNAi. **a, e**, SMC-1 immunoprecipitation enriched for TIM-1, but its association with the cohesin complex was more salt-sensitive (it dissociated at 0.5 M KCl or more) than core cohesin subunits (**e**). **f**, Reciprocal TIM-1 immunoprecipitation confirmed the cohesin interaction. **g**, Northern blots with a genomic DNA probe spanning exons 6 and 7 detected a single 5.5-kb *tim-1* mRNA transcript. **h**, Estimate of REC-8 protein concentrations in *rec-8*(RNAi) animals; the values below the gels are REC-8 concentrations normalized to MIX-1 and compared to wild-type animals.

molecular mass 150,000 (M_r 150K). In addition, a significant number of peptide fragments matched the tryptic peptides predicted for worm ORF Y75B8A.22, which was originally named *tim-1* (ref. 10) on the basis of its similarity to *Drosophila timeless*, a regulator of circadian rhythm.

Using TIM-1-specific antibodies (Fig. 1d and Methods) we confirmed the association of TIM-1 with cohesin. Both SMC-1 and SCC-1 immunoprecipitations were specifically enriched in TIM-1 (Fig. 1a, e, and data not shown). The reciprocal TIM-1 immunoprecipitation precipitated SMC-1, SMC-3 and SCC-3 (Fig. 1f). More stringent wash conditions (at least 0.5 M KCl) dissociated some TIM-1 from the cohesin complex, but the precipitated core cohesin complex was stable at 1 M KCl without TIM-1 (Fig. 1e). Thus, TIM-1 associates specifically with cohesin but is less tightly connected than other subunits.

The connection between TIM-1 and cohesin was unexpected, given the function of *Drosophila timeless* in the perpetuation and entrainment of circadian rhythm through its interaction with the clock gene *period* (*per*)^{11,12}. However, several recent discoveries hinted that *timeless* homologues have an essential function that is independent of circadian rhythm regulation. *Drosophila* has a *timeless* paralogue called *timeout*, which more strongly resembles the single *C. elegans* and mammalian homologues than does *timeless*^{13,14}. The function of *Drosophila timeout* is unknown, but knockout of the mouse *mTim1* gene causes early embryonic lethality¹⁴, whereas *timeless* mutant flies are fully viable. Finally, cryptochromes (mCry1/2) fulfil functions in mammalian circadian rhythm provided by TIMELESS in flies¹². The connection between *C. elegans* TIM-1 and cohesin provides a clue to the likely ancient and conserved role for *timeless/timeout* homologues in metazoans.

To assess the function of TIM-1 *in vivo*, we determined the subcellular location of TIM-1 and examined whether TIM-1 depletion affected *C. elegans* viability and chromosome segregation. TIM-1 and cohesin have similar cell-cycle-dependent patterns of subcellular localization during mitosis in somatic and germline tissues (Supplementary Fig. 1b). At interphase, TIM-1 and cohesin subunits accumulate most intensely and have a diffuse nuclear appearance. By the metaphase–anaphase transition, concentrations of both are greatly diminished and the proteins seem to be excluded from the compacted DNA. The disruption of either *tim-1* or individual cohesin genes by RNA-mediated interference (RNAi) caused complete embryonic lethality (Supplementary Table 2). Most nuclei of dying embryos had two 5S ribosomal DNA (rDNA) fluorescence *in situ* hybridization (FISH) signals (Fig. 2a), indicating normal chromosome segregation. Occasional nuclei had more than two FISH signals, perhaps from the premature dissociation of sister chromatids (Fig. 2a). However, nuclei from embryos treated with double RNAi against *him-1* and either *scc-1* or *tim-1* had numerous FISH signals, indicating severe chromosome segregation defects and extensive aneuploidy (Fig. 2a). By comparison, RNAi to *tim-1* only (Fig. 2b) caused aberrant chromosome segregation during germline mitosis that resembled the defective segregation in *him-1(h55)* (Fig. 2b) or *him-1(h134)* (Supplementary Fig. 1a, c) null mutants (see Methods). These results suggest a role for TIM-1 and other *timeless* paralogues in the establishment and/or maintenance of sister-chromatid cohesion in metazoans.

The role of TIM-1 in chromosome cohesion can be resolved further by analysing *tim-1* mutant defects in meiosis, where cohesion and other cohesin-dependent functions are essential for homologous chromosome synapsis, recombination and segregation¹⁵. We show below that TIM-1 has an essential function in meiotic chromosome cohesion by means of its regulation of cohesin subunits.

A description of wild-type meiotic chromosome behaviour provides the context for interpreting *tim-1* meiotic defects. Each *C. elegans* gonad arm contains hundreds of syncytial germline nuclei arranged along the distal–proximal axis in a spatial progression that

follows the temporal progression into and through meiotic prophase (Fig. 3a)¹⁶. Nuclei entering meiotic prophase undergo a marked spatial reorganization that partitions chromosomes and nucleoli to opposite sides (Fig. 3a, c)¹⁷. In this 'transition zone', the equivalent of leptotene/zygotene, the chromatin has a crescent-shaped appearance, and homologues initiate pairing¹⁸ by a process that is independent of mature synaptonemal complex (SC)^{7,19}, an ordered protein structure that holds homologues together. By pachytene, homologous chromosomes redistribute about the nuclear periphery and achieve full alignment and synapsis by means of SC-dependent processes^{18,19}. The synapsed homologues (Fig. 3a–c)¹⁷ are proficient in crossover recombination and appear as closely aligned, parallel tracks that stain with 4,6-diamidino-2-phenylindole (DAPI) (Fig. 3b) and have a single 5S rDNA FISH signal (Fig. 3e, f). Chromosomes become desynapsed in diplotene/diakinesis owing to disassembly of the SC. During diakinesis, the homologues are held together by transient physical linkages (chiasmata), the result of crossover recombination and chromosome cohesion distal to the crossover. Wild-type homologues form six axially oriented bivalents.

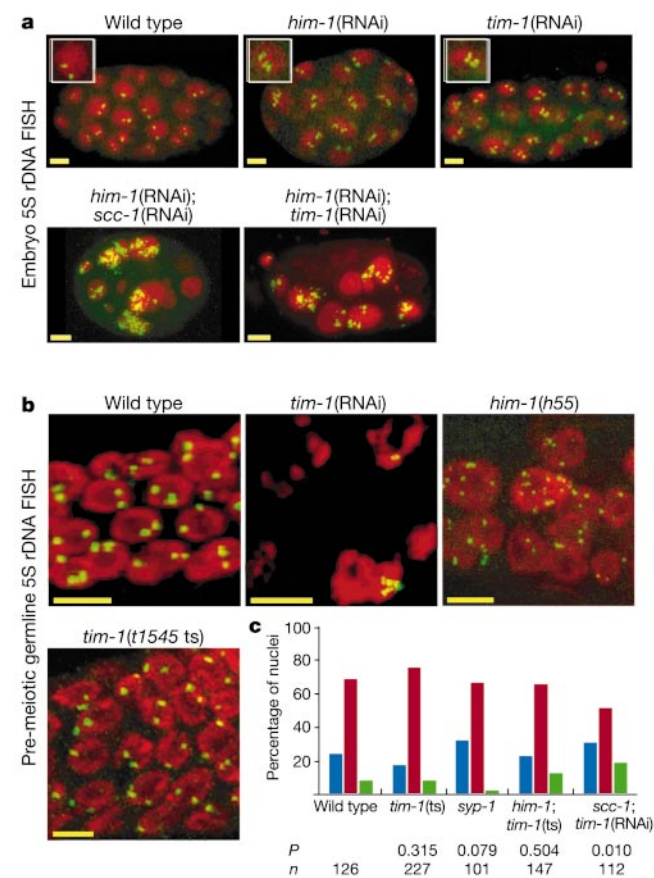


Figure 2 TIM-1, like cohesin subunits, is essential for mitotic chromosome segregation in somatic and germline tissues. **a**, The DNA ploidy of untreated and RNAi-treated embryos was assayed by FISH to 5S rDNA: red, DNA; green, FISH fluorescence. Single RNAi treatment targeting either *tim-1* or *him-1* caused occasional nuclei with more than two FISH signals but most nuclei of dying embryos appeared wild-type, having at most two signals (upper panels). In contrast, severe aneuploidy resulted from double RNAi treatments targeting *him-1* and either *scc-1* or *tim-1* (lower panels). **b**, In pre-meiotic nuclei, *tim-1*(RNAi) and *him-1* null mutations caused aneuploidy, as assayed by FISH. In contrast, pre-meiotic nuclei from *tim-1*(t1545ts) and wild-type animals grown at 25 °C were indistinguishable (**b, c**). Scale bars, 5 μ m. **c**, Graph showing the distribution of FISH signals in pre-meiotic nuclei of different genotypes: blue, one signal; red, two signals; green, more than two signals. *n* is the total number of nuclei scored; *P* is calculated relative to wild type.

We used the *tim-1(t1545ts)* allele to assess TIM-1 function in meiotic chromosome cohesion, because it selectively disrupts meiosis but not germline mitosis under a specific temperature-shift regime (Fig. 2b, c, and Methods). Thus, the meiotic defects in *tim-1(ts)* nuclei cannot be attributed to defective germline mitosis.

Imaging of DAPI-stained *tim-1(ts)* mutant nuclei revealed meiotic defects that resembled those caused by RNAi depletion of REC-8,

a meiosis-specific cohesin subunit that replaces the mitosis-specific SCC-1 subunit⁷. Transition-zone nuclei of *tim-1(ts)* or *rec-8(RNAi)* mutants had normal polarized organization (Fig. 3c), and a similar proportion of paired and unpaired homologues to that in wild-type nuclei, on the basis of 5S rDNA FISH signals (Fig. 3j and ref. 7). However, both *tim-1(ts)* and *rec-8(RNAi)* mutants had an extended transition zone (Fig. 3c) in which polarized nuclei persisted into the

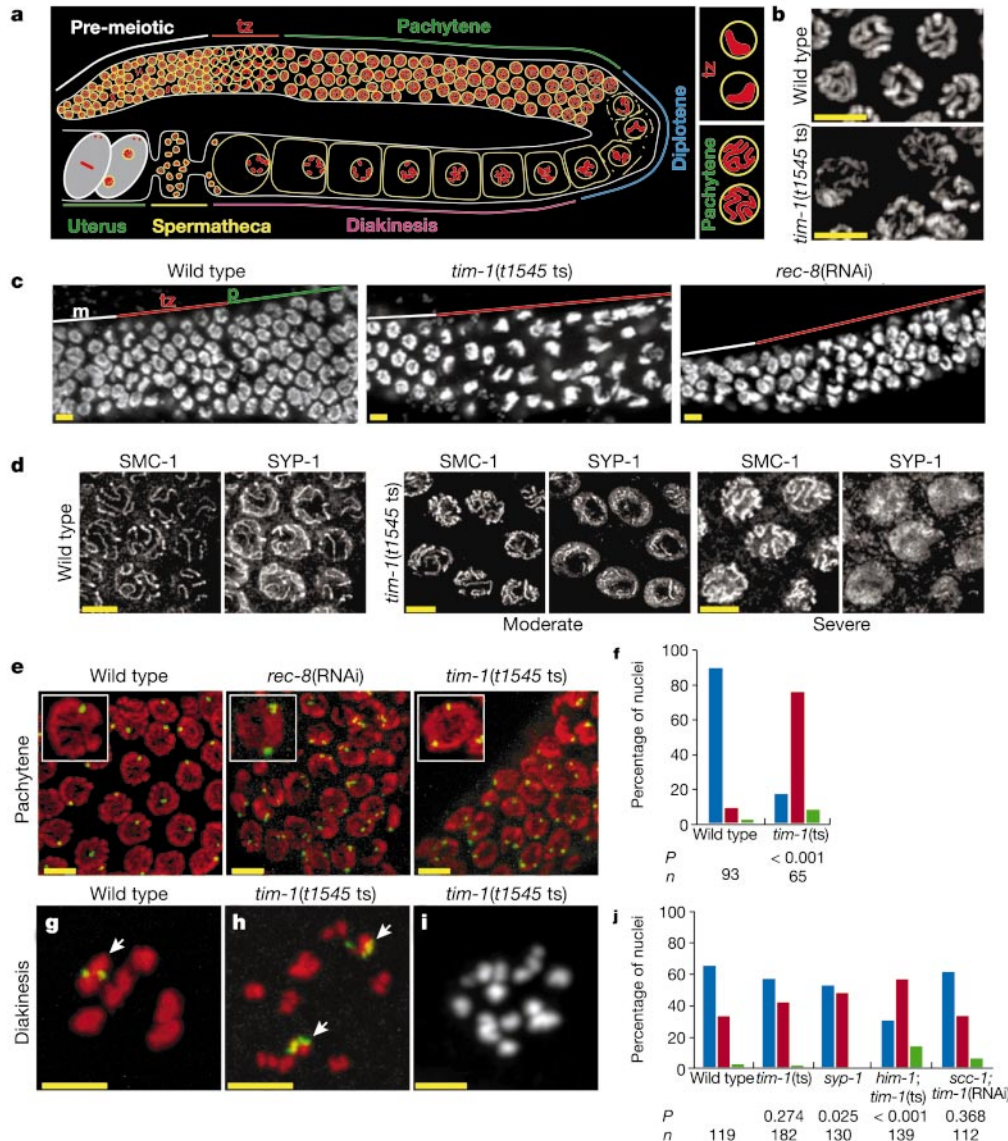


Figure 3 *tim-1* mutants are defective in meiotic chromosome cohesion. **a**, Schematic drawing of an adult hermaphrodite gonad arm: pre-meiotic region (mitotic germ cell proliferation and pre-meiotic DNA replication), transition zone (tz; homologue pairing and initiation of synapsis), pachytene (completion of synapsis and crossover recombination), diplotene (SC disassembly) and diakinesis (homologue association maintained by chiasmata and chromosome cohesion). **b**, Pachytene chromosomes in *tim-1(t1545ts)* mutants fail to achieve lengthwise alignment typical of synapsed wild-type pachytene chromosomes and appear as single, rather than parallel, DAPI-stained tracks. **c**, DAPI-stained image of a wild-type gonad shows the normal progression of nuclei from mitosis (m), through two stages of meiotic prophase, the transition zone (also known as leptotene/zygotene) and pachytene (p). *tim-1(ts)* and *rec-8(RNAi)* mutants exhibit normal nuclear reorganization and homologue pairing in the transition zone, but the redistribution of the chromosomes toward the nuclear periphery that normally accompanies the onset of pachytene is delayed, causing an extended transition zone. **d**, In *tim-1(ts)* gonads, SC central region component SYP-1 (ref. 19) is localized to short discontinuous patches rather than along the entirety of homologous chromosomes, consistent with a defect in SC

assembly. The co-staining marker SMC-1 is unaffected by the *tim-1* mutation. **e**, The two 5S rDNA FISH signals in *tim-1(ts)* pachytene-region nuclei reveal asynapsis of chromosome V homologues. **f**, Quantification of **e** for late-pachytene nuclei: blue, one FISH signal per nucleus; red, two signals; green, more than two signals. **g**, In a wild-type diakinesis nucleus, chromosome V homologues are physically linked in a bivalent (white arrow). **h**, In the *tim-1(ts)* mutant nuclei, chromosome V homologues are prematurely separated (white arrows). **i**, Evidence for sister chromatid separation: more than 12 DAPI-stained bodies are apparent. Scale bars, 5 μ m. **j**, Quantification of pairing of transition-zone nuclei. *him-1(RNAi);tim-1(ts)* mutants showed significantly decreased pairing of the 5S rDNA locus in transition-zone nuclei compared with wild-type or *syp-1(me17)* mutant animals (see also Supplementary Table 3), indicating that synapsis-independent homologue pairing requires cohesin. Most pre-meiotic nuclei in all three sets of animals had the expected number of FISH signals (one or two), indicating few, if any, mitotic chromosome abnormalities. In contrast, double RNAi of *scc-1* and *tim-1* had negligible effects on pairing of the 5S rDNA locus in transition-zone nuclei, despite its disruption to the pre-meiotic germline (Fig. 2c).

region of the gonad that is normally filled with pachytene nuclei. We reasoned that the extended transition zone might result from disrupted SC assembly due to defective cohesin function. Rec8 is required for the normal formation of the yeast SC²⁰, and disruption of the *C. elegans* SC by depleting the SC central-region component SYP-1 causes an extended transition zone¹⁹. Indeed, SYP-1 localization was aberrant (Fig. 3d) in *tim-1(ts)* mutants. SYP-1 assembled in short discontinuous patches along chromosomes of most *tim-1(ts)* pachytene-region nuclei (85%, $n = 62$), rather than continuously along homologous chromosomes, as in wild-type nuclei. In contrast, the co-staining control marker SMC-1 localized properly. The degree of SYP-1 disruption was directly correlated with the severity of the extended transition zone (Fig. 3d). These results provide functional evidence for the involvement of TIM-1 in SC formation, perhaps through its effect on cohesin function.

Although meiotic chromosomes of *tim-1(ts)* or *rec-8(RNAi)* mutants reorganized into a pachytene-like arrangement just before diplotene (Fig. 3b, c), like those of *syp-1* mutants, they failed to achieve the normal lengthwise alignment typical of synapsed pachytene chromosomes (Fig. 3b). Only 17% ($n = 65$) of *tim-1(ts)* nuclei within the late pachytene region had one 5S rDNA FISH signal, in comparison with 89% ($n = 93$) of wild-type nuclei, indicating a severe defect in synapsis (Fig. 3e, f), an outcome expected from aberrant chromosome cohesion.

Homologous chromosomes of *tim-1(ts)* mutants rarely formed chiasmata. Of 142 diakinesis nuclei from wild-type germlines (Fig. 3g), all had six bivalents. Instead, 101 of 105 *tim-1(ts)* nuclei had 7–12 staining bodies, with an average of 10; they were mostly univalents and an occasional bivalent, indicating disrupted homologue linkage (Fig. 3h). Three nuclei had more than 12 bodies (Fig. 3i), implying both homologue and sister chromatid separation, consistent with a disruption of meiotic chromosome cohesion. *rec-8(RNAi)* (ref. 7, data not shown) and *him-1(ts)* (Supplementary Fig. 1d) produced similar results. The partial separation of *tim-1(ts)* sister chromatids probably reflects the incomplete disruption of TIM-1 activity by the temperature-sensitive mutation. Together, the meiotic defects caused by the disruption of *tim-1*—the extended transition zone correlated with mislocalized SYP-1, asynapsis in pachytene, and the lack of chiasmata in diakinesis—mimic the defects caused by a disruption of meiotic cohesin, providing compelling evidence for the essential role of *tim-1* in meiotic chromosome cohesion.

TIM-1 could act directly with cohesin throughout meiotic prophase to promote chromosome cohesion, or it could facilitate cohesin stability or localization to chromatin, acting only before meiotic prophase. To evaluate these models, we first defined the *C. elegans* meiotic cohesin subunits and compared their germline localization with that of TIM-1. In many organisms, meiosis-specific cohesin subunits replace their mitotic paralogues¹⁵. Although the *C. elegans* REC-8 protein substitutes for its mitotic SCC-1 paralogue⁷, the other mitotic cohesin subunits have no worm paralogues, making their replacement in meiosis unlikely.

Consistent with this premise is the observation that SMC-1, SMC-3 and SCC-3 recapitulate the meiotic localization pattern of REC-8. All four proteins associate with the chromatin of transition-zone nuclei (Fig. 4a, b, and data not shown), which is consistent with the loading of meiotic cohesin before or during pre-meiotic S phase. SMC-1 co-localizes with REC-8 in pachytene nuclei, and all four proteins assemble along the longitudinal axes of synapsed chromosomes (Fig. 4c). Moreover, two findings show that SMC-1 localization to pachytene chromosomes does not require chromosomes to have a pairing partner and SC. In XO males, SMC-1 antibody staining is evident along the unpaired X chromosome (Fig. 4d). In XO males carrying a IV:X fusion chromosome (*mnt12*), in which IV is paired and X is unpaired, SMC-1 localizes along the entirety of the fusion chromosome (Fig. 4d). Finally, SMC-1 co-localizes with REC-8 in diakinesis, and SMC-1, SMC-3

and REC-8 all adopt a cruciform pattern (Fig. 4e), which is consistent with a role for these proteins in maintaining connections between homologues and between sister chromatids. In contrast, SCC-3 localized throughout the chromatin, indicating a possible additional role for SCC-3 in diakinesis not played by other cohesins (Fig. 4f).

TIM-1 protein localization differed remarkably from that of meiotic cohesin, suggesting that TIM-1 facilitates the loading or stability of cohesin proteins, rather than serving as an integral complex component. TIM-1 was diffusely distributed throughout pre-meiotic nuclei and disappeared abruptly as nuclei entered meiotic prophase (Fig. 4a, b). Unlike cohesin subunits, TIM-1 was not detected in transition-zone or pachytene nuclei, but reappeared in diplotene and accumulated in diakinesis. TIM-1 was excluded from chromosomes in diakinesis and probably accumulates in oocytes only for its subsequent function in embryonic mitosis (Fig. 4g).

To determine whether TIM-1 affects cohesin subunits, we examined the distribution of REC-8, SCC-3, SMC-1 and SMC-3 in *tim-1* mutants. REC-8 was not detectable on *tim-1(ts)* (Fig. 5a–c) or *tim-1(RNAi)* chromosomes (data not shown) in meiotic prophase, although it was present in pre-meiotic nuclei (Fig. 5a), indicating that TIM-1 affects the stability or loading of REC-8 but not its production. REC-8 depletion did not alter TIM-1 localization in pre-meiotic nuclei (data not shown), which is consistent with TIM-1's regulating REC-8. In *tim-1* mutants, SCC-3 concentrations were variably reduced, and in half the gonads the residual protein was undetectable on the meiotic chromosomes (Fig. 5a–c). In contrast, SMC-1 seemed properly localized to meiotic chromosomes of *tim-1(ts)* (Fig. 5a–c) or *tim-1(RNAi)* mutant gonads (data not shown), suggesting that a loss of TIM-1 function does not affect SMC localization. TIM-1 therefore differs from the yeast Scc2/4 cohesin regulatory proteins, which affect the loading of both SMC and non-SMC cohesin components²¹, and differs from yeast Pds5, which persists with the cohesin complex^{22,23}.

If SMC-1 and SMC-3 load onto chromatin independently of non-SMC proteins, as suggested by our data, then disruption of REC-8 should not affect SMC protein localization. Indeed, severe depletion of *rec-8* by RNAi (Fig. 1h) caused no detectable change in SMC-1 or SMC-3 localization but did cause mislocalization of SCC-3 (Fig. 5d, e, and Supplementary Fig. 1e). SCC-3 mislocalization is consistent with structural studies in budding yeast showing that Scc1 mediates the interaction between Scc3 and the Smc1/Smc3 heterodimer²⁴. Furthermore, SMC-1 and SMC-3 localization seemed unperturbed under more stringent conditions in which *rec-8(RNAi)* treatment was performed in *tim-1(ts)* mutants (Fig. 5f). These results indicate that TIM-1 promotes meiotic chromosome cohesion through its regulation of cohesin subunits and that TIM-1 might preferentially regulate the loading or stability of REC-8 and SCC-3 before leptotene/zygotene. These results also show the differential loading or retention of individual cohesin subunits at the onset of meiosis.

The presence of SMC proteins on *tim-1(ts); rec-8(RNAi)* mutant chromosomes raised the possibility that cohesion activity might persist, thus obscuring the complete role of cohesin in meiosis. Reducing SMC-1 concentrations with RNAi in a *tim-1(ts)* mutant revealed the involvement of cohesin in the early pairing of homologues before synapsis. Although the chromosomes of *him-1(RNAi); tim-1(ts)* animals underwent the initial spatial reorganization typical during leptotene/zygotene, only 30% of chromosome V homologues paired at the 5S rDNA locus in comparison with 65% in untreated animals, reflecting a 54% decrease in pairing (Fig. 3j, Supplementary Table 3).

This defect in early pairing is unlikely to be a reflection of aberrant germline mitosis, because the pre-meiotic regions of *him-1(RNAi); tim-1(ts)* and wild-type animals were indistinguishable by FISH analysis (Fig. 2c, and Supplementary Table 3).

Moreover, the simultaneous disruption of *tim-1* and the mitotic cohesin gene *scc-1* by RNAi failed to disrupt pairing at the 5S rDNA locus (Fig. 3j, and Supplementary Table 3), although it did disrupt germline mitosis (Fig. 2c).

The defect in pairing cannot be attributed to the disruption of SYP-1 and hence the SC, because pairing at the 5S rDNA locus was more severely disrupted in *him-1(RNAi);tim-1(ts)* mutants than in *syp-1* mutants (30% versus 52%, a 42% decrease in pairing; Fig. 3j, and Supplementary Table 3). These results show that synapsis-

independent homologue pairing relies in part on cohesin function, thus revealing a crucial role for cohesin not previously demonstrated in metazoans. These experiments probably underestimate the contribution of cohesin to pairing, because efforts to minimize defects in pre-meiotic chromosome segregation required that cohesin function be disrupted only partly.

Our work has revealed unexpected roles in chromosome segregation for the *C. elegans* homologue of the *Drosophila* *timeless* gene, a key regulator of circadian rhythm. *C. elegans* TIM-1 interacts

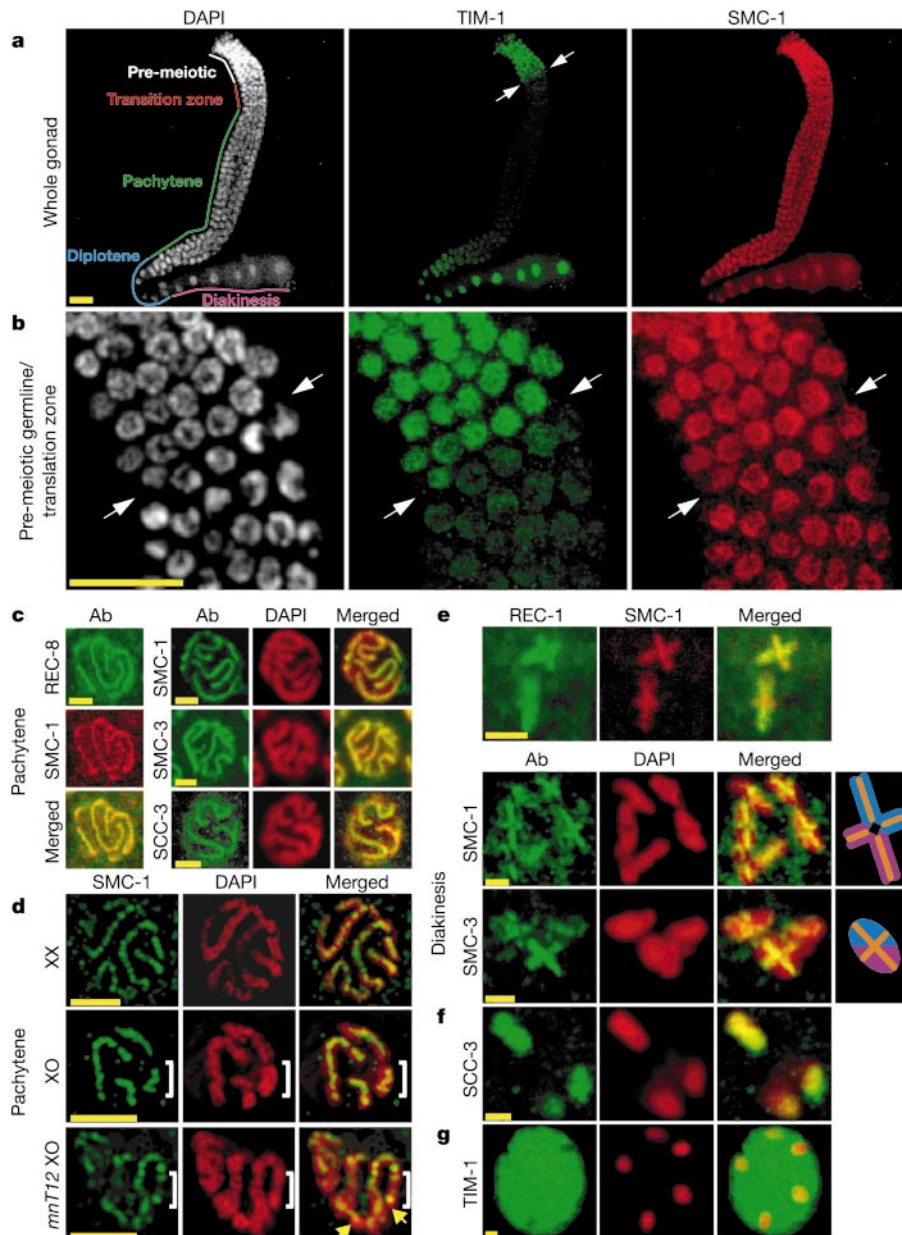


Figure 4 TIM-1 is restricted to pre-meiotic nuclei, unlike meiotic cohesin, which localizes between sister chromatids throughout meiotic prophase. **a**, **b**, Antibody and DAPI fluorescence images of dissected wild-type gonads. Intense nuclear TIM-1 accumulation observed in pre-meiotic nuclei was reduced abruptly as nuclei entered meiosis. The boundary between the pre-meiotic region and the transition zone (opposing white arrows) is characterized by crescent-shaped chromatin (**b**). **c**, SMC-1, SMC-3 and SCC-3 proteins co-localize with REC-8 along the length of pachytene chromosomes. **d**, Anti-SMC antibody staining along the unpaired male X chromosome (dense DAPI staining, white brackets) of wild-type XO males and those with the *mnT12(IV;X)* fusion chromosome

shows that SMC-1 localization is not dependent on homologous chromosome pairing and SC formation. *mnT12* chromosome ends are marked by yellow arrows. **e**, SMC-1, SMC-3 and REC-8 have identical cruciform staining patterns, consistent with their function in the meiotic cohesin complex. At the right is a diagram of the cohesin staining pattern in early and late diakinesis bivalents showing cohesin (orange) and sister chromatid pairs (blue and purple). **f**, SCC-3 localizes to the interface of sister chromatids and homologous chromosomes and also throughout the bivalent, indicative of additional functions. **g**, By diakinesis, maternally loaded TIM-1 fills the nucleoplasm but is excluded from DNA. Scale bars, 20 μ m (**a**, **b**); 2 μ m (**c**–**g**).

physically with the cohesin complex and thereby mediates chromosome cohesion. Not only is TIM-1 critical for mitotic chromosome segregation in somatic and germline tissues, it is also essential for chromosome segregation in meiosis. Through its crucial role in localizing non-SMC cohesin subunits to chromatin, before or

during the pre-meiotic S phase, TIM-1 has a fundamental role in meiosis, stabilizing homologous chromosome associations during synapsis and sister chromatid cohesion in diplotene/diakinesis. This differential effect of TIM-1 on the non-SMC subunits suggests the independent loading or stabilization of cohesin components and indicates that SMC proteins might retain partial function without the context of the entire cohesin complex.

Given the vital role of *C. elegans tim-1* in chromosome cohesion and the essential function of mouse *mTim1* in embryo viability, we suggest that the evolution of the involvement of *timeless* in the *Drosophila* circadian rhythm resulted from a duplication and subsequent specialization of the fly *timeout* gene, which more closely resembles the single paralogue present in *C. elegans*, mice and humans. Comparison of predicted protein structures reinforces this view. *Drosophila* TIMEOUT, *C. elegans* TIM-1 and mouse *mTim1* all have contiguous HEAT/Armadillo (Arm) repeats that reside within a similar region of each protein (Supplementary Fig. 2). In contrast, the HEAT/Arm repeats of *Drosophila* TIMELESS are interrupted by one of two PERIOD-interaction domains²⁵, indicating that this change could enhance the association between PERIOD and TIMELESS. Given the unique structure of *Drosophila* TIMELESS and the conclusions of our present study, we propose that chromosome cohesion, rather than circadian rhythm regulation, is the ancient and conserved function for *timeless* paralogues in metazoans. □

Methods

him-1 encodes SMC-1

Deletion mapping of the SMC-1 ORF F28B3.7 by polymerase chain reaction (PCR) placed the gene under deletion *sDf4* but not *hDf6*, in an interval harbouring five genes with lethal null phenotypes, including the likeliest candidate *him-1*. The temperature-sensitive *him-1*(e879) allele and two genetic *him-1* null alleles, *h55* and *h134*, have been described previously^{5,26}. A 9.3-kilobase (kb) genomic PCR product covering F28B3.7 rescued the sterility of *him-1*(h55) mutants, and DNA sequence changes were identified for all three *him-1* alleles, indicating that *him-1* encodes the Smc1 homologue. The *him-1*(h55) null allele carries a deletion that removes portions of the second exon and intron. The deletion is predicted to disrupt the splicing of the second and third exons and probably causes a frame shift that aborts translation after amino acid 250. The *him-1*(h134) null allele causes a C-T transition at nucleotide 3271 of the coding sequence. This mutation generates a premature stop codon predicted to truncate 172 amino acids from the carboxy terminus. The *him-1*(e879) partial loss-of-function allele results in the P1138S substitution. cDNA sequencing of *him-1* agrees with the predicted intron-exon boundaries for F28B3.7 (GenBank accession no. 17506950) with the exception of intron 7, where the actual 3' splice site is 57 nucleotides downstream of the predicted 3' splice site.

Antibody production

The following antigens were used to generate antibodies: C-terminal peptides to DPY-27, MIX-1, SMC-3 and TIM-1; amino-terminal peptide to SCC-3; SMC-1 residues 652–1075 fused to a His₆ tag; SCC-1 residues 207–500 and TIM-1 residues 286–1001 fused to glutathione S transferase tags. TIM-1 peptide antibodies recognize a protein of *M_r* 150K from wild-type extracts but no protein from extracts of animals treated with RNAi against *tim-1* (Fig. 1d), thus establishing antibody specificity. The antibodies also detected a *tim-1* RNAi-sensitive product of *M_r* 120K (Fig. 1d), which seems to be a TIM-1 post-lysis cleavage product, consistent with (1) the single 5.5-kb *tim-1* transcript identified on northern blots (Fig. 1g) and (2) the DNA sequence of *tim-1* cDNA and products from PCR with reverse transcription. Immunofluorescence staining was imaged by confocal microscopy²⁷. Chromosomes in Fig. 4d were imaged on an API DeltaVision deconvolution microscope system.

Worm lysates, embryonic extracts and immunoprecipitation

Whole worm lysates were prepared from at least 30 animals washed with PBS and boiled in SDS sample buffer with 3 M urea. Embryo extract preparation and immunoprecipitation were performed as described²⁸.

Genetics

Gene function was disrupted by injecting double-stranded RNA into wild-type (N2), *unc-32*(e189) *tim-1*(t1545ts)/*qC1* [*dpy-19*(e1259) *glp-1*(q339)] III or *unc-32*(e189) *tim-1*(t1545)/*hT2* III strains. *tim-1*(t1545ts) homozygotes are maternal-effect lethal at both 20 and 25 °C (ref. 29), and maternally rescued animals (*m⁺/z⁻*) exhibit meiotic defects when grown at 25 °C. For analysis of *tim-1*(t1545ts) meiotic phenotypes, embryos were collected at 20 °C from *tim-1* heterozygotes and allowed to develop into adults at 25 °C; maternally rescued (*m⁺/z⁻*) *tim-1* homozygotes were isolated by picking Unc hermaphrodites. The allele *t1545* was originally described as a maternal-effect lethal mutation in the gene *csg-5* (ref. 29). It was subsequently shown to be a temperature-sensitive *tim-1* allele (M.J. and A.E.R., unpublished observations).

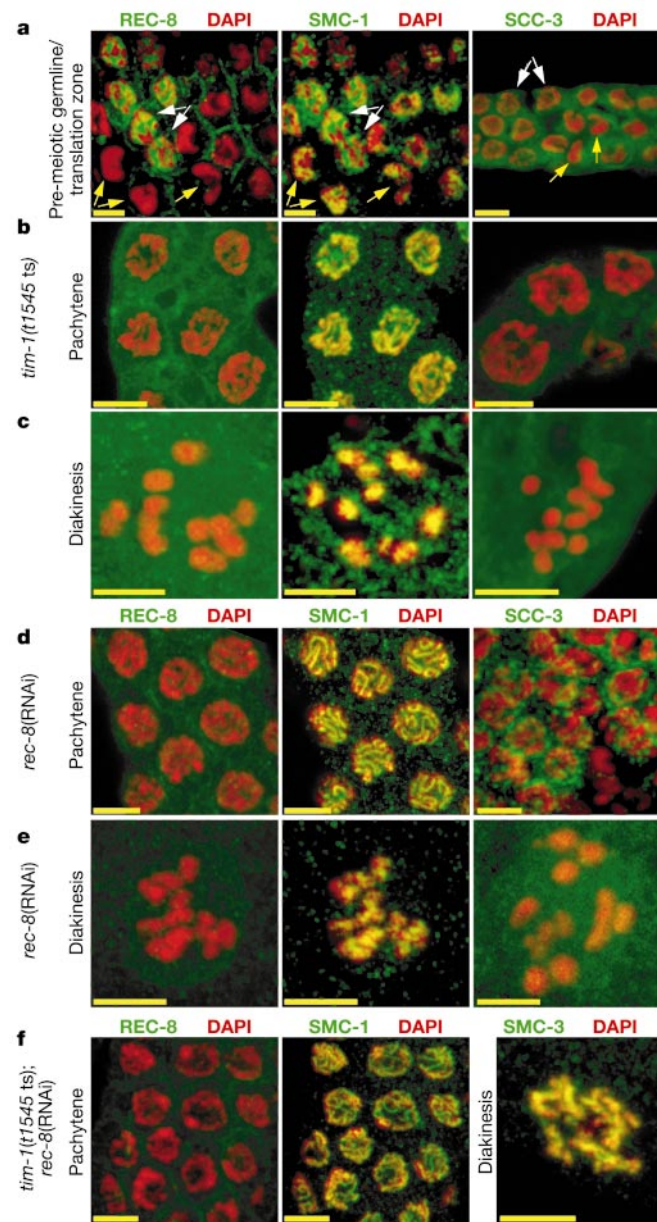


Figure 5 Loss of *tim-1* function preferentially disrupts the loading or stability of non-SMC cohesin subunits. Immunofluorescence photomicrographs of cohesin subunits in *tim-1*(ts) (a–c), *rec-8*(RNAi) (d, e) and *tim-1*(ts);*rec-8*(RNAi) (f) mutant germlines. **a**, accumulation of SMC-1, REC-8 and SCC-3 is unperturbed in pre-meiotic nuclei (white arrowheads) of *tim-1*(ts) mutants. However, on transition-zone chromatin (yellow arrowheads) of *tim-1*(ts) mutants, REC-8 staining is undetectable and SCC-3 staining is severely reduced, but SMC-1 staining is normal (also Fig. 3d). **b, c**, Similarly, REC-8 and SCC-3 staining is severely disrupted in pachytene (**b**) and diakinesis (**c**) chromosomes of *tim-1*(ts) mutants, but SMC-1 staining is normal. **d, e**, Unlike SCC-3 staining, SMC-1 staining (and SMC-3; Supplementary Fig. 1e) on pachytene (**d**) and diakinesis (**e**) chromosomes is unaffected by *rec-8*(RNAi), showing the stable chromatin association of cohesin SMC subunits with few or no non-SMC components (see Fig. 1h for quantification of the REC-8 depletion). **f**, In *tim-1*(ts);*rec-8*(RNAi) double mutants, SMC-1 and SMC-3 localize to chromosomes in pachytene (see also Supplementary Fig. 1e) and diakinesis. Note that diakinesis chromosomes often fail to resolve in the double mutants. Scale bars, 5 μm.

mnT12(IV;X)/+ males were generated by crossing homozygous *mnT12* hermaphrodites with wild-type males.

Double-stranded RNA interference

Templates for *him-1*, *scc-1*, *scc-3*, *rec-8* and *tim-1* RNA transcription were generated by PCR amplification from N2 genomic DNA with the use of the primer sets listed in Supplementary Table 4. The *smc-3* template was amplified by PCR using T3 and T7 primers from the Kohara cDNA *yk361c10*. Double-stranded RNA (1–5 mg ml⁻¹) was injected into the gonads of young gravid hermaphrodites.

For immunostaining and FISH studies the following RNAi-treated animals were scored: (1) *rec-8*(RNAi), F₁ progeny from injected animals; (2) *scc-1*(RNAi);*tim-1*(RNAi) or *him-1*(RNAi);*tim-1*(RNAi), injected parents dissected 48 h after injection; (3) *him-1*(RNAi);*tim-1*(ts), injected *tim-1*(ts) adults (grown at 25 °C as above) dissected 24 h after injection; (4) *him-1*(RNAi), *tim-1*(RNAi), *him-1*(RNAi);*scc-1*(RNAi) and *him-1*(RNAi);*tim-1*(RNAi), injected parents dissected 24 h after injection. FISH analysis was performed on embryos dissected from adults 48 h after injection with double-stranded RNA from the targeted gene(s).

FISH and statistical analysis

FISH with 5S rDNA was performed as described²⁷. The number of FISH signals per nucleus was counted for the mitotic region (50 µm or less from the distal tip), transition zone (30 µm or less from the first appearance of crescent-shaped DAPI figures) and pachytene (40 µm or less distal to diplotene). The nuclei were distributed into five categories: one, two, three, four and more than four FISH signals per nucleus. The statistical comparisons of FISH signals in mutant and wild-type nuclei were performed on the categories of one, two and more than two FISH signals per nucleus by pairwise χ^2 tests. For the transition-zone nuclei analysed in Fig. 3j, the distribution of FISH signals in the population of nuclei for each genotype reflected the distribution of FISH signals in each individual animal scored.

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Cks1-dependent proteasome recruitment and activation of *CDC20* transcription in budding yeast

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Cks proteins are small evolutionarily conserved proteins that interact genetically and physically with cyclin-dependent kinases. However, in spite of a large body of genetic, biochemical and structural research, no compelling unifying model of their functions has emerged^{1,2}. Here we show, by investigating the essential role of Cks1 in *Saccharomyces cerevisiae*, that the protein is primarily involved in promoting mitosis by modulating the transcriptional activation of the APC/C protein–ubiquitin ligase activator Cdc20. Cks1 is required for both the periodic dissociation of Cdc28 kinase from the *CDC20* promoter and the periodic association of the proteasome with the promoter. We propose that the essential role of Cks1 is to recruit the proteasome to, and/or dissociate the Cdc28 kinase from, the *CDC20* promoter, thus facilitating transcription by remodelling transcriptional complexes or chromatin associated with the *CDC20* gene.

cks1-35 cells arrest in metaphase with high levels of the anaphase inhibitor securin, known as Pds1 in *S. cerevisiae*^{3,4}. Mitotic progression of wild-type, unperturbed cells is regulated primarily by the accumulation of mitotic cyclins^{5–7} and activation of the protein–ubiquitin ligase APC/C by its substrate-specific activator Cdc20. This enables the proteolysis of Pds1 and the onset of anaphase^{8–10}. The isolation of *CDC20* as a strong multicopy suppressor of a *cks1* temperature-sensitive mutant (this study; data not shown)

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prompted us to investigate whether *cks1* mutants were defective in the function or regulation of *CDC20*. Expression of *CDC20* was monitored in the absence of functional Cks1 in a time course of synchronized cells (Fig. 1a–c). The expression of *CDC20* mRNA is normally periodic and peaks before the metaphase–anaphase transition¹¹. However, in *cks1-35* cells *CDC20* mRNA and Cdc20 protein are expressed only at a constitutive, basal level (Fig. 1a,b and data not shown), indicating that the metaphase block observed in *cks1* mutants might be primarily due to impaired *CDC20* expression.

If the primary defect of *cks1* mutants is an impaired expression of *CDC20*, constitutive expression of *CDC20* should rescue the loss of Cks1 function. To test this possibility, a *cks1* disruption mutant was transformed with an episomal high-copy plasmid containing the genomic *CDC20* locus. As can be seen in Fig. 1d, high copy expression of *CDC20* was able to significantly rescue the growth defect conferred by disruption of *CKS1*, presumably because the basal level of expression of *CDC20* from multiple copies of the gene can compensate for the inability of the *cks1* disruptant to induce *CDC20* transcription. Enabling *CDC20* transcription is therefore the growth-limiting function of Cks1.

To determine whether Cks1 interacts directly with the promoter region of *CDC20* (Fig. 2a), chromatin immunoprecipitation (ChIP) assays were performed. Haemagglutinin (HA)-tagged Cks1 efficiently immunoprecipitated the *CDC20* promoter region from extracts prepared from asynchronous wild-type cultures to an extent similar to Myc-tagged Mcm1 and HA-tagged Ndd1 (Fig. 2b), two transcription factors that have previously been found to regulate other genes expressed in M phase, including *CLB2*, *SWI5* and *CDC5* (refs 12–15) and are expected to bind the promoter of *CDC20* because of the presence of consensus Mcm1 and SFF/FKH sites, respectively (Fig. 2a). Consistent with Cks1's being a cyclin-dependent-kinase-binding protein was the observation that HA-tagged Cdc28 (Cdk1) could also immunoprecipitate the promoter region of *CDC20* (Fig. 2b).

To determine whether binding of Cdc28 to the promoter of *CDC20* is dependent on functional Cks1, we compared the ability of HA-tagged Cdc28 and HA-tagged Cdc28-1N to immunoprecipitate the *CDC20* promoter in cells grown at the restrictive temperature (37 °C). *cdc28-1N* encodes a protein with a greatly reduced ability to bind Cks1 (ref. 16). To exclude the possibility of cell-cycle effects, the experiment was performed with HA-tagged alleles of Cdc28 and Cdc28-1N expressed from centromeric plasmids in a background also expressing chromosomal wild-type Cdc28. Although there is no cell-cycle arrest under these conditions, we observed that Cdc28-1N consistently immunoprecipitated the *CDC20* promoter more efficiently than did wild-type Cdc28 (Fig. 2c). These data indicate that Cks1 might be required to dissociate Cdc28 from the *CDC20* promoter.

To determine whether the interactions between Cks1, Cdc28 and the *CDC20* promoter are cell-cycle dependent, cells progressing through the cell cycle synchronously were processed for ChIP analysis (Fig. 2d). HA-tagged Cks1 and Cdc28 immunoprecipitated the promoter region of *CDC20* periodically, although with different kinetics. Maximal association of Cks1 with the *CDC20* promoter occurred when *CDC20* mRNA accumulation was also maximal. In contrast, periodic association of Cdc28 with the promoter preceded the expression of *CDC20*, and declined to a minimum when *CDC20* mRNA levels were maximal. Finally we examined whether the periodic association and dissociation of Cdc28 was dependent on its association with Cks1: Cdc28-1N was inefficiently removed from the promoter of *CDC20* and only after a long delay (Fig. 2e). In this experiment the epitope-tagged alleles were again expressed from plasmids in cells also expressing the chromosomal wild-type *CDC28* gene. These data are consistent with the observation that the *cdc28-1N* mutation is partly dominant over wild-type *CDC28* in that heterozygotes are delayed for nuclear division (see Supplementary

Fig. S1), and therefore indicate that periodic and robust expression of *CDC20* is dependent on the interaction of Cks1 with Cdc28, which in turn is required to mediate periodic dissociation of the latter from the promoter of *CDC20*.

Cks1 has previously been shown to interact genetically and physically with the proteasome⁴. Components of the proteasome were assayed for association with the *CDC20* promoter and for the dependence of this association on functional Cks1. HA-tagged Rpn3/Sun2, Rpt1/Cim5 (non-ATPase and ATPase components of the 19S regulatory particle, respectively) and Pre1 (proteasomal subunit $\beta 4$ of the 20S catalytic particle) co-precipitated the promoter of *CDC20* in asynchronous wild-type cells similarly to Cks1 (Fig. 3a,b). However, as shown in Fig. 3b, binding of both Rpt1 and Pre1 with the promoter of *CDC20* was significantly decreased in the *cks1-35* mutant at the restrictive temperature (37 °C), indicating a requirement for Cks1 function. Finally, we determined that binding of the 19S and 20S proteasomal components was specific to the promoter of *CDC20* (Fig. 3c), because Rpt1 and Pre1 could not co-precipitate the promoters of the cell-cycle-regulated genes *CLB2*, *CLN1* and *CLN2*. In contrast, both Rpt1 and Pre1 efficiently precipitated the promoters of *PUR5* and *GAL1*, shown previously to be regulated by the proteasome^{17,18}. Interestingly, proteasomal

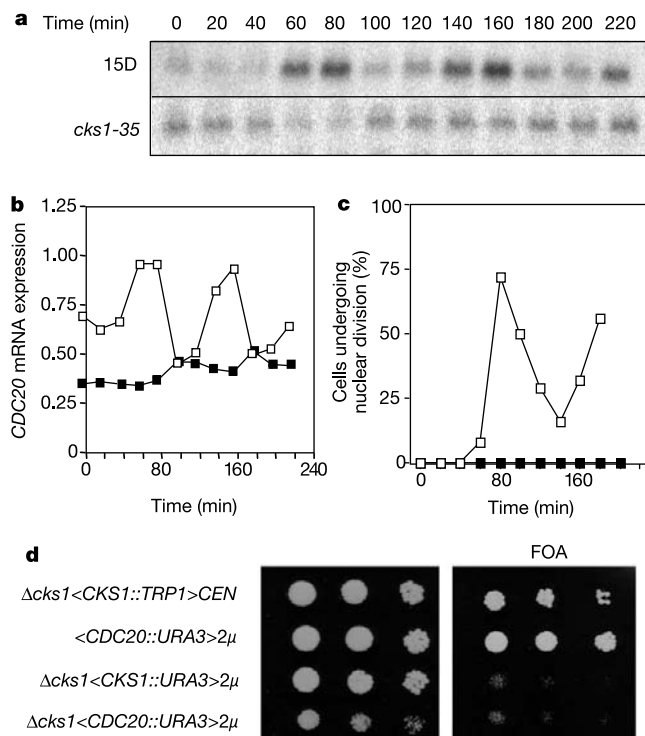


Figure 1 Cks1 is involved in the transcriptional regulation of *CDC20*. **a**, RNA prepared from samples of 15D (wild-type) and *cks1-35* cells collected every 20 min in a time-course experiment at 37 °C, was analysed by northern blotting after synchronization with α -factor. Blots were sequentially hybridized with radiolabelled probes corresponding to *CDC20* and *ACT1*. **b**, Normalization of the *CDC20* signal to the actin signal. Open squares, 15D cells; filled squares, *cks1-35* cells. **c**, Kinetics of nuclear division scored by DAPI staining of nuclei of 15D cells (open squares) and *cks1-35* cells (filled squares). **d**, Congenic strains of genotypes *cks1::LEU2 < CKS1::TRP1 > CEN < CDC20::URA3 > 2 μ* , *cks1::LEU2 < CKS1::TRP1 > CEN*, *cks1::LEU2 < CKS1::URA3 > 2 μ* and *cks1::LEU2 < CDC20::URA3 > 2 μ* were spotted at increasing dilutions (2,000, 400 and 80 cells from left to right, respectively) on dextrose-supplemented minimal plates and dextrose-supplemented minimal medium containing 5-fluoroorotic acid (FOA) and incubated at 34 °C for 48 h.

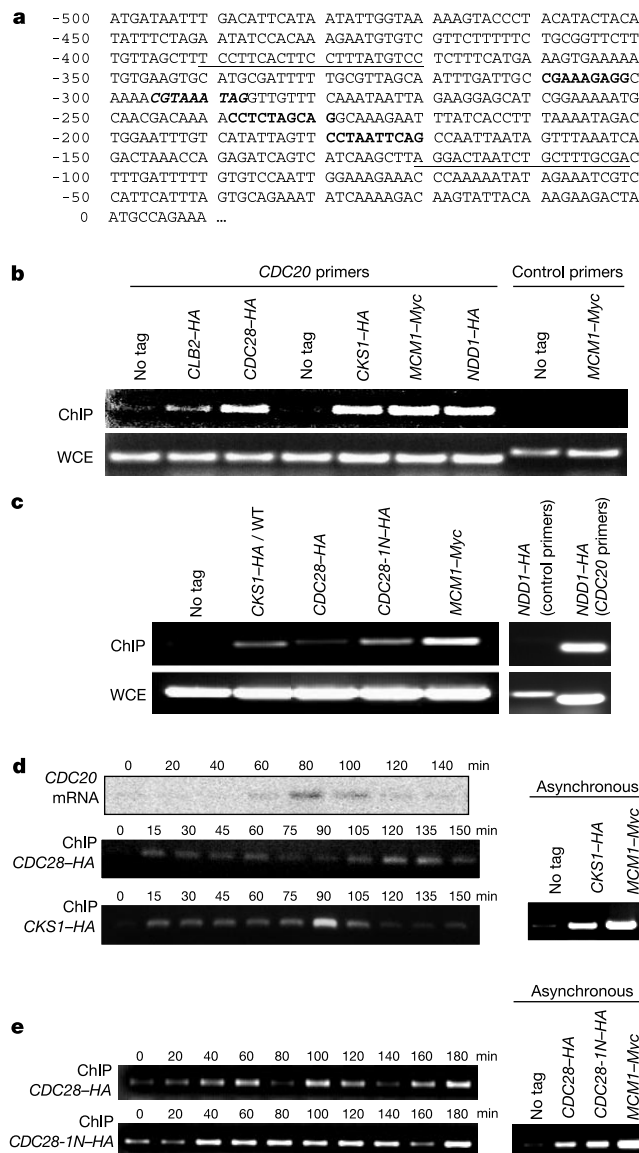


Figure 2 Cks1 associates with the upstream activating sequence (UAS)/promoter of *CDC20*. **a**, The 500-base-pair (bp) sequence upstream of the open reading frame (ORF) of *CDC20* encompassing its presumptive promoter. Primers used for polymerase chain reaction (PCR) amplification of the 300-bp fragment in the ChIP assays are underlined. The three consensus Mcm1-binding sites are in bold. The consensus SFF-binding site is in bold italics. The first ATG of the ORF lies at position 1. **b**, Cks1 associates with the UAS/promoter region of *CDC20*. Asynchronous cultures of the wild-type 15D strain (no tag) or of 15D expressing HA-tagged Cks1, HA-tagged Clb2, HA-tagged Cdc28, Myc-tagged Mcm1 or HA-tagged Ndd1 were immunoprecipitated with anti-HA or anti-Myc antibodies and processed for ChIP assays as described previously²³. WCE, whole cell extract. **c**, Relationship of Cdc28 binding to Cks1 function. Cultures expressing HA-tagged Cdc28 or HA-tagged Cdc28-1N (both expressed from a centromeric plasmid in a background expressing wild-type Cdc28) were grown for 3 h at the restrictive temperature (37 °C) and processed for ChIP assays. PCRs were performed as in **b**; a negative control (alternative primers) is shown for HA-tagged Ndd1. WT, wild type. **d**, Cell-cycle dependence of Cks1 and Cdc28 binding. Cultures expressing HA-tagged Cks1 or HA-tagged Cdc28 were synchronized with α -factor and released into YEPG medium at 37 °C. ChIP analysis was performed as in **b**; a typical northern blot of *CDC20* mRNA expression in 15D cells grown in similar experimental conditions and collected every 20 min is shown for comparison. **e**, Cell-cycle dependence of Cdc28 and Cdc28-1N binding to the *CDC20* promoter. Cultures ectopically expressing HA-tagged Cdc28 or HA-tagged Cdc28-1N in a wild-type *CDC28* background were synchronized with α -factor and released into YEPG medium at 37 °C. ChIP analysis was performed as in **b**.

binding to the *PUR5* and *GAL1* promoters was also found to be dependent on Cks1 (Fig. 3b). These results confirm that the proteasome associates efficiently only with a specific subset of genes, one of which is *CDC20*, and also indicate that Cks1 function might be required for proteasomal recruitment at several proteasome-dependent transcription units. We next determined whether association of the proteasome with the *CDC20* promoter is cell-cycle regulated (Fig. 3d, and data not shown). Indeed, Rpt1 and Pre1 associate with the *CDC20* promoter with kinetics similar to that observed for Cks1. That the peak of proteasome binding corresponds to the reproducible cell-cycle peak of *CDC20* transcription (80 min) is consistent with the idea that proteasome recruitment is required for optimal *CDC20* transcription.

Cell-cycle analysis of *CDC20* transcription in a strain carrying the *sug1-25* mutation of ATPase subunit Rpt6 (ref. 17), revealed a strong dependence on Rpt6 (Fig. 4a,b). The delayed kinetics and markedly decreased levels of *CDC20* transcript relative to wild-type cells coincided with a significant delay and loss of synchrony of nuclear division in *sug1-25* cells (Fig. 4c). To determine whether the mitotic

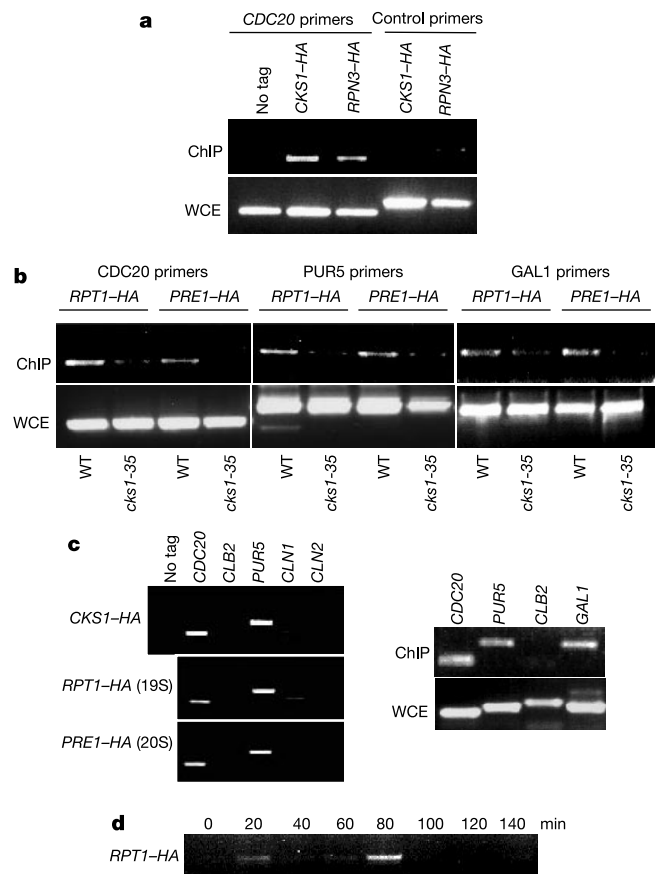


Figure 3 Components of the 19S and 20S proteasome particles interact with the promoter of *CDC20*. **a**, Asynchronous cultures expressing HA-tagged Cks1 or HA-tagged Rpn3/Sun2 were processed for ChIP analysis. PCRs were performed with the primers described in Fig. 2. WCE, whole cell extract. **b**, Cultures expressing HA-tagged Pre1 or HA-tagged Rpt1/Cim5 in wild-type (WT) or *cks1-35* backgrounds were grown in YEPD medium, then shifted to the restrictive temperature (37 °C) for 3 h and processed for ChIP analysis as in Fig. 2. **c**, Asynchronous cultures expressing HA-tagged Cks1, HA-tagged Rpt1/Cim5 or HA-tagged Pre1 were processed for ChIP analysis. PCRs were performed with primers to amplify a region within the promoter of *CDC20*, *CLB2*, *PUR5*, *CLN1*, *CLN2* or *GAL1*. **d**, Cell-cycle dependence of Rpt1 binding. Wild-type cultures expressing HA-tagged Rpt1 were synchronized with α -factor for 3 h and released into YEPG medium at 37 °C. ChIP analysis was performed as in Fig. 2.

delay in *sug1-25* cells is specifically due to the defect in *CDC20* transcription, we expressed *CDC20* ectopically in mutant and congenic wild-type cells. *CDC20* driven from the *GAL1* promoter could partly rescue the delay and asynchrony of nuclear division in *sug1-25* cells, whereas no change in kinetics of nuclear division was observed in wild-type cells ectopically expressing *CDC20* (Fig. 4d,e).

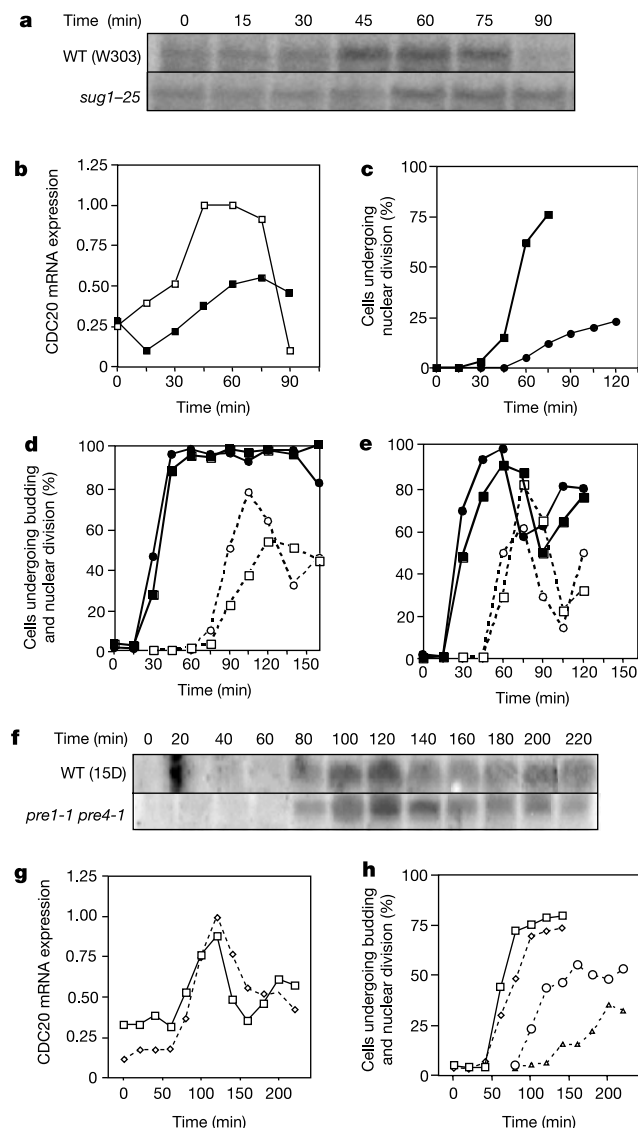


Figure 4 Proteasomal function is essential for *CDC20* transcription. **a**, RNA was prepared from samples of W303a and W303a *sug1-25* cells at 36 °C after synchronization with α -factor. Northern blotting was performed with radiolabelled probe corresponding to *CDC20*. WT, wild type. **b**, Quantification of the *CDC20* signal. Open squares, W303a cells; filled squares, W303a *sug1-25* cells. **c**, Kinetics of nuclear division scored by DAPI staining of nuclei of W303a cells (squares) and W303a *sug1-25* cells (circles) after synchronization with α -factor. **d**, Kinetics of budding and nuclear division of *sug1-25* cells ectopically expressing *CDC20*. Filled squares, vector budding; filled circles, *CDC20* budding; open squares, vector nuclear division; open circles, *CDC20* nuclear division. **e**, Kinetics of budding and nuclear division of W303 cells ectopically expressing *CDC20*. Symbols as in **d**. **f**, RNA was prepared from samples of 15d and 15d *pre1 pre4* cells at 38 °C after synchronization with α -factor. Northern blotting was performed sequentially with radiolabelled probe corresponding to *CDC20* and *ACT1* (encoding actin). **g**, Quantification of *CDC20* signal normalized to *ACT1*. Squares, 15d cells; diamonds, 15d *pre1 pre4* cells. **h**, Kinetics of budding and nuclear division. Squares, wild-type budding; diamonds, *pre1 pre4* budding; circles, wild-type nuclear division; triangles, *pre1 pre4* nuclear division.

The partial rescue of the *sug1-25* strain is most probably due to the inefficient expression of *CDC20* in this strain, which is significantly lower than the endogenous level in wild-type cells (see Supplementary Fig. S2). To address the issue of whether a functional 19S proteasome is required for the cell-cycle-dependent removal of Cdc28 from the *CDC20* promoter, we performed Cdc28 ChIP experiments on synchronized *sug1-25* and wild-type cells. The removal of Cdc28 from the *CDC20* promoter was delayed (40 min versus 60–80 min) and less robust in the *sug1-25* mutant (see Supplementary Fig. S3).

The experiments described above establish a role for the proteasome in *CDC20* transcription but do not distinguish between a proteolytic and a non-proteolytic role. We therefore analysed mutants defective in proteasome protease function¹⁹ (*pre1 pre4* double mutant) for *CDC20* transcript levels as a function of cell cycle progression. Mutant cells exhibited a strong delay in nuclear division (Fig. 4g,h), presumably owing to defects in proteolysis of M-phase targets. However, transcription of *CDC20* was not affected (Fig. 4f), indicating that the proteasomal function required might not be proteolytic.

Genetic and physical interactions reported here and previously⁴ suggest that the Cks1 function is most likely to be linked to proteasomal function, although the exact nature of the relationship between Cks1 and the proteasome remains to be determined. It has been suggested that the ATPases of the 19S particle might be employed in the remodelling of protein complexes in roles other than proteolysis^{17,18}. In particular, the *sug1-25* allele of *RPT6* employed in this study is defective in transcriptional elongation of the *PUR5* gene, whereas mutations on the 20S proteasome that eliminate protease activity confer no effect¹⁷. Along the same lines, a complex comprising the 19S particle, but not subunits from the 20S proteolytic particle, has recently been shown to be recruited to the *GAL1-10* promoter by the Gal4 transactivator¹⁸.

For *CDC20* transcription, we cannot rule out a role for the 20S particle, especially because a subunit of the 20S particle, Pre1, was precipitated with the promoter of *CDC20*. However, it is interesting to note that, on the basis of our data, Pre1 also localizes to the promoter of *PUR5* and *GAL1*, where it is also not required for transcriptional functions¹⁷. Nevertheless, on the basis of experiments using a combination of 20S particle mutants, *pre1* and *pre4*, a proteolytic role for the 20S particle seems unlikely. We propose that Cks1 is initially recruited to the *CDC20* promoter together with Cdc28. Under these conditions, *CDC20* is not transcriptionally active. Cks1 then facilitates an exchange reaction in which the proteasome is bound and Cdc28 is released, promoting transcription. Whether the essential function of the proteasome is to remove Cdc28 or to facilitate transcription directly, or both, remains to be determined. □

Methods

Yeast strains and growth conditions

All strains used in this study are listed in Supplementary Table S1 together with their relevant genotypes. Strains were constructed and grown in accordance with standard procedures²⁰. For synchrony experiments, cells were synchronized for 3 h with 40 ng ml⁻¹, 1 μ g ml⁻¹ and 3 μ g ml⁻¹ α -factor at 23 °C, for the strains 15Daub²¹, 15Daub²¹ and W303, respectively, followed by washing twice and centrifugation to remove the pheromone. Cells were then cultured in preheated fresh rich medium and aliquots were removed at fixed intervals for northern blot analysis, ChIP analysis or scoring for nuclear division by staining with 4,6-diamidino-2-phenylindole (DAPI). For experiments with a galactose-inducible *CDC20* construct, incubation with α -factor (10 μ g ml⁻¹) was in YEP medium containing 2% raffinose for 3 h at 30 °C, after which cells were washed and resuspended in heated YEP medium containing 2% raffinose and 2% galactose.

Northern blotting

Total RNA isolation and northern blotting were performed as described²². For details see Supplementary Information.

Western blotting

Exponential-phase cultures ($D_{600} = 0.4$) of cells with the expression of *myc18-CDC20* under the control of either the *CDC20* (PY581), *GAL1^Δ* or *GAL1* promoter were induced

by the addition of galactose to 2%. An attenuated *GAL1* promoter was used to express *myc18-CDC20* in the W303 control to achieve levels close to that of the *sug1-25* mutant, which is defective in *GAL1* induction. Cells were left to grow for 1.5 h at 36 °C, then harvested. Cells were lysed with glass beads in 25 mM Tris-HCl pH 8.0, 2 mM EDTA, 1 µg ml⁻¹ leupeptin, 1 µg ml⁻¹ pepstatin, 2 µg ml⁻¹ aprotinin, 2 mM phenylmethylsulphonyl fluoride. Protein concentrations in soluble lysates were measured with Lowry assays against a standard curve of BSA. Protein samples (100 µg) were resolved on a Tris-glycine SDS 8% polyacrylamide gel, transferred to poly(vinylidene difluoride) membrane, probed with 9E10 anti-Myc primary antibody and horseradish-peroxidase-conjugated goat anti-mouse secondary antibody.

ChIP experiments

ChIP experiments were performed essentially as described in ref. 23 and references therein. For additional details, see Supplementary Information.

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POT1 as a terminal transducer of TRF1 telomere length control

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Human telomere maintenance is essential for the protection of chromosome ends, and changes in telomere length have been implicated in ageing and cancer^{1–4}. Human telomere length is regulated by the TTAGGG-repeat-binding protein TRF1 and its interacting partners tankyrase 1, TIN2 and PINX1 (refs 5–9). As the TRF1 complex binds to the duplex DNA of the telomere, it is unclear how it can affect telomerase, which acts on the single-stranded 3' telomeric overhang. Here we show that the TRF1 complex interacts with a single-stranded telomeric DNA-binding protein—protection of telomeres 1 (POT1)—and that human POT1 controls telomerase-mediated telomere elongation. The presence of POT1 on telomeres was diminished when the amount of single-stranded DNA was reduced. Furthermore, POT1 binding was regulated by the TRF1 complex in response to telomere length. A mutant form of POT1 lacking the DNA-binding domain abrogated TRF1-mediated control of telomere length, and induced rapid and extensive telomere elongation. We propose that the interaction between the TRF1 complex and POT1 affects the loading of POT1 on the single-stranded telomeric DNA, thus transmitting information about telomere length to the telomere terminus, where telomerase is regulated.

Telomeric DNA can be maintained by telomerase, which uses the 3' end of the telomeric overhang as a substrate. In germline and tumour cells, the length of the duplex telomeric repeat array is kept within a narrow range by telomere length homeostasis. Extensive evidence indicates that the length of human telomeres is evaluated at each individual chromosome end based on the amount of TRF1 complex bound along the length of the telomere^{5–8,10–14}. An analogous *cis*-acting 'protein counting' mechanism of the duplex telomere-binding protein Rap1 (ref. 15) has been proposed for the control of telomere length in budding yeast. However, it is not clear how regulatory proteins engaged on the duplex part of the telomere affect telomerase at the 3' end of the telomeric overhang. In yeast, the 3' overhang is bound by Cdc13, which recruits and regulates telomerase¹⁶, but there is no known connection between Cdc13 and Rap1. Human cells contain a candidate overhang-binding protein, POT1, which is related to Cdc13 and other oligosaccharide/oligonucleotide-binding (OB) fold proteins with single-stranded telomeric DNA-binding activity^{17–20}. Our data suggest that interactions between POT1 and the TRF1 complex allow POT1 to transduce information about telomere length to the telomere terminus, where the regulation of telomerase takes place.

To study the association of POT1 and other proteins with telomeres, we developed a chromatin immunoprecipitation (ChIP) assay, in which protein-associated telomeric DNA is detected by quantitative hybridization of a TTAGGG repeat probe to DNA dot blots (Fig. 1a–c; see also Supplementary Fig. 1). Alu sequences are used as a negative control in each experiment (Fig. 1c). As a second negative control, ChIPs were performed with various pre-immune sera, which yielded background signals (Fig. 1a–c). By contrast, TRF1 ChIPs yielded about 30% (28 ± 4; *n* = 7) of the total telomeric DNA in the HT1080-derived cell line HTC75 (data not shown). Similar telomeric DNA yields were obtained in TRF1 ChIPs in a variety of cell strains, including GM847, HeLa, IMR90 and BJ cells (Fig. 1 and data not shown). As the ChIP values are presented as a percentage of the total telomeric DNA, they are

corrected for telomere length variations in different cell lines. The telomeric association of TRF1-interacting protein 2 (TIN2) and components of the TRF2 complex was also readily detectable by ChIP. The yield of telomeric DNA was approximately 15% for TRF2 and its interacting partner human RAP1 (Fig. 1), and 10–15% for TIN2 (see below). Two rabbit anti-peptide antibodies to POT1 as well as two sera raised against the full-length protein immunoprecipitated a significant fraction (about 10%) of the telomeric DNA in ChIPs from HTC75 and GM847 cells (Fig. 1; see also Supplementary Fig. 1). Consistent with this finding, two of these sera (1048 and 1049) also detected POT1 at telomeres by immunofluorescence (Supplementary Fig. 1). Furthermore, telomeric DNA was recovered in ChIPs with a Myc antibody in cells expressing Myc-POT1 (Fig. 1a). Thus, POT1 behaves as a telomeric protein on the basis of ChIP and immunofluorescence.

As POT1 binds to single-stranded telomeric TTAGGG repeats but not to duplex telomeric DNA *in vitro*^{17,18} (D.L., H. Parsons, K. Hoke and T.d.L., unpublished data), we anticipated that the telomeric binding of POT1 requires the presence of the 3' telomeric overhang. To test this mode of binding *in vivo*, we made use of the fact that inhibition of the telomere protective factor TRF2 results in loss of 50% of the single-stranded telomeric DNA but leaves the duplex

part of the telomere intact²¹. TRF2 was inhibited using two doxycyclin-regulated cell lines (T4 and T19) that express the TRF2(Δ B Δ M) dominant-negative allele of TRF2 (ref. 21). As expected, induction of TRF2(Δ B Δ M) resulted in a significant reduction (twofold, $P = 0.027$) in the binding of TRF2 to telomeres as assessed by ChIP (Fig. 1b, c). Furthermore, there was an associated loss of the TRF2-interacting factor RAP1 from the telomeres (sixfold reduction, $P = 0.009$; Fig. 1b, c) and about 50% loss of the 3' overhang (data not shown). The amount of POT1 at telomeres was also reduced at least twofold in cells expressing TRF2(Δ B Δ M) compared with uninduced controls (Wilcoxon two-sample test, $P = 0.013$; Fig. 1b, c). As a control, TRF1 was not significantly affected by inhibition of TRF2 (Fig. 1b, c; see also ref. 21). Thus, the diminished POT1 binding to telomeres correlated with a reduction in the amount of single-stranded telomeric DNA. The lack of interaction with TRF2 and RAP1 (see below) argues against the possibility that TRF2(Δ B Δ M) removed POT1 from telomeres through protein interaction. However, we cannot rule out an indirect effect of the perturbation of the whole telomeric structure on POT1 binding.

Biochemical studies showed that the amino-terminal OB fold of POT1 is required for DNA binding^{17,18} (D.L., H. Parsons, K. Hoke, J. Donigian and T.d.L., unpublished data). To test the role of the POT1 OB fold in telomere binding, we examined the localization of a mutant lacking the N-terminal 127 amino acids (POT1(Δ OB); Fig. 2a). Myc-tagged full-length POT1 and POT1(Δ OB) yielded proteins of the predicted molecular mass detectable with anti-Myc sera as well as with two antibodies raised against a POT1 peptide (Fig. 2b). Although the anti-peptide antibodies should detect four of the five proposed splice variants of POT1 (ref. 18), only a single endogenous protein was detected by both sera in HTC75 and HeLa cells and this polypeptide had an apparent molecular mass consistent with full-length POT1 (Fig. 2b and data not shown). Moderate overexpression of exogenous Myc-POT1 and Myc-POT1(Δ OB) resulted in nearly complete suppression of endogenous POT1 protein detectable by immunoblotting (Fig. 2b); the mechanism of this downregulation is not known. Immunofluorescence showed that POT1(Δ OB) still had the ability to localize to telomeres (Fig. 2c). This result was unexpected as we anticipated the DNA-binding domain of POT1 to be required for targeting to telomeres. We confirmed the telomeric association of POT1(Δ OB) using the ChIP assay (Fig. 2d, e). In these experiments, ChIPs with POT1 antibodies using cells expressing Myc-POT1(Δ OB) or Myc-tagged full-length POT1 resulted in the same yield of approximately 7% of the telomeric DNA. ChIPs with the Myc antibody were less efficient, but still yielded a significant fraction of telomeric DNA (about 2.5%) even when Myc-tagged POT1 lacked the OB fold (Fig. 2d, e). Although variations in expression levels interfere with direct comparison between the telomeric association of POT1(Δ OB) and full-length POT1, the data indicate that POT1(Δ OB) can still locate to telomeres. Owing to the very low abundance of endogenous POT1 in cells expressing POT1(Δ OB), it is unlikely that the telomeric association of POT1(Δ OB) occurs through an interaction with the endogenous full-length protein. Hence, the telomere localization of POT1(Δ OB) suggested that direct DNA binding mediated by the N-terminal OB fold is not the only mode of recruitment of POT1 to telomeres.

Therefore, we explored the possibility that POT1 interacts with the telomeric protein complexes formed by TRF1 and TRF2. Co-immunoprecipitation experiments showed that TRF1 associated with Myc-tagged POT1 (Fig. 3a), and immunoprecipitation of endogenous POT1 resulted in recovery of the TRF1-interacting factors TIN2 and tankyrase 1 (Fig. 3b–d). In parallel experiments, no interaction was detected between POT1 and TRF2 or its interacting partners RAP1 or Mre11 (refs 22, 23) (Fig. 3a, b; see also Supplementary Fig. 2), indicating that the association of POT1 with the TRF1 complex was highly specific. The association of POT1 with

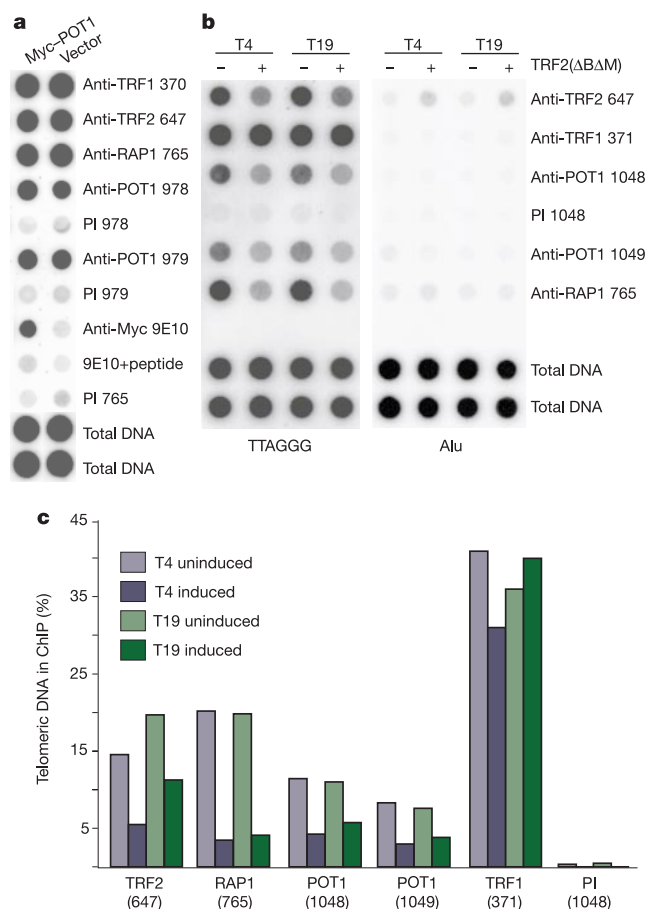


Figure 1 Telomeric association of POT1 correlates with single-stranded DNA (ssDNA). **a**, Telomeric ChIP on GM847 cells infected with pLPC or pLPC-Myc-POT1 using the indicated antibodies or pre-immune serum (PI). Dot blots were hybridized with a TTAGGG repeat probe. **b**, ChIP after reduction in telomeric overhang DNA owing to inhibition of TRF2. HTC75 clones T4 and T19 were induced (+) to express TRF2(Δ B Δ M) for 7 days and were processed alongside uninduced cells (–). Duplicate dot blots were probed for telomeric or Alu repeats. **c**, Quantification of the data in **b** representing per cent TTAGGG repeat DNA recovered in each ChIP. Averaged duplicate signals obtained with total DNA samples were used as 100% value for the quantification.

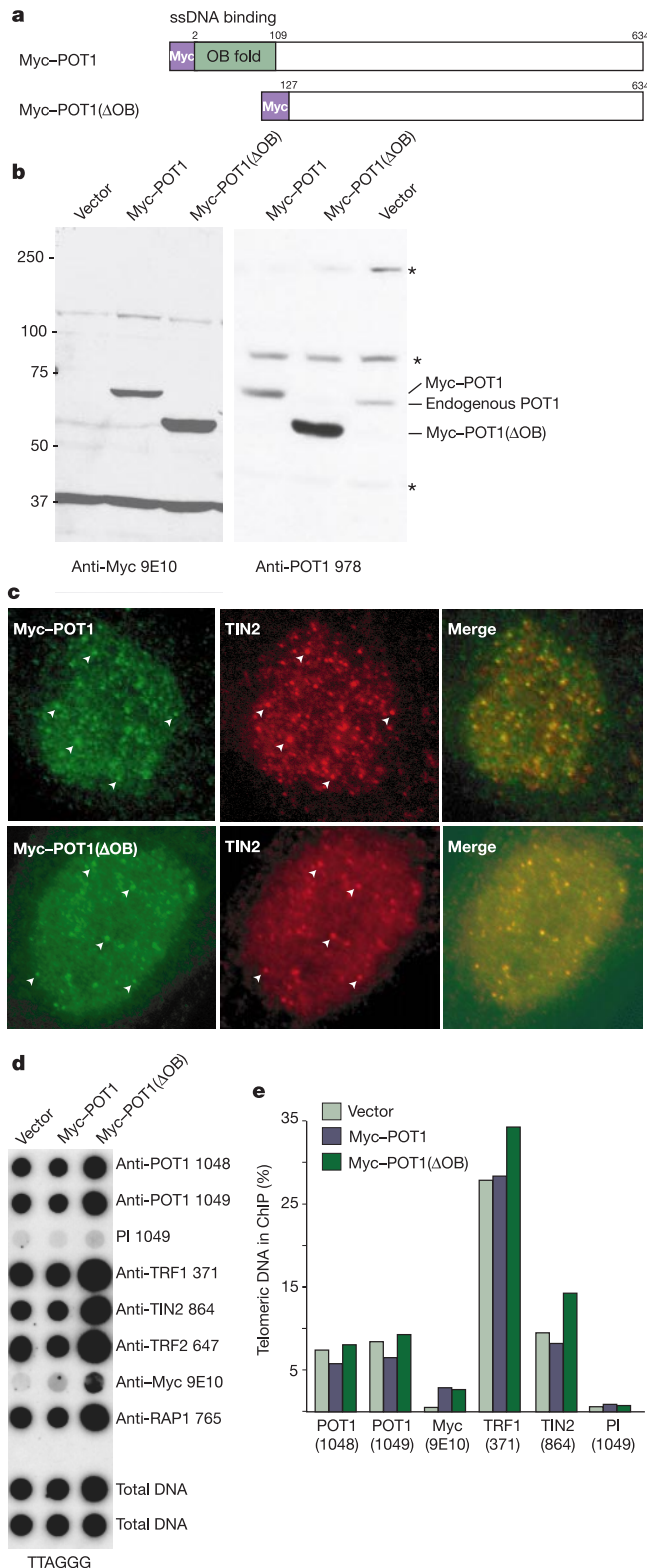


Figure 2 Telomeric association of POT1(ΔOB). **a**, POT1(ΔOB). **b**, Immunoblot of HTC75 cells infected with Myc-POT1, Myc-POT1(ΔOB) or pLPC probed for Myc or POT1. Asterisks indicate nonspecific bands (not detected by duplicate POT1 serum 979). Molecular mass standards (kDa) are shown on the left-hand side. **c**, Myc (9E10) and TIN2 (864) immuno fluorescence images of IMR90 cells expressing Myc-POT1 or Myc-POT1(ΔOB). Arrowheads indicate examples of POT1 localizing together with TIN2. Both POT1 forms show non-telomeric signals. **d**, ChIPs on HTC75 cells containing the indicated retroviruses. Owing to telomere elongation over 150 population doublings, the TTAGGG signals are 3.5-fold higher for Myc-POT1(ΔOB). **e**, Quantification of the data in **d** (as in Fig. 1c). As the histograms represent the percentage of input telomeric DNA, they are corrected for telomere length changes.

the TRF1 complex was not dependent on the OB fold, as Myc-tagged POT1(ΔOB) was recovered in association with both TRF1 and TIN2 (Fig. 3a, b). Thus, the binding of POT1(ΔOB) to telomeres could be explained by the interaction of POT1 with the TRF1 complex.

To test whether the accumulation of POT1 on telomeres depended on its interaction with the TRF1 complex, we used tankyrase 1 overexpression, which is the most effective method for removing the TRF1 complex from telomeres⁷. Poly(ADP-ribosylation) of TRF1 by the PARP activity of tankyrase 1 results in loss of its DNA-binding activity *in vitro*²⁴, and this effect is thought to be

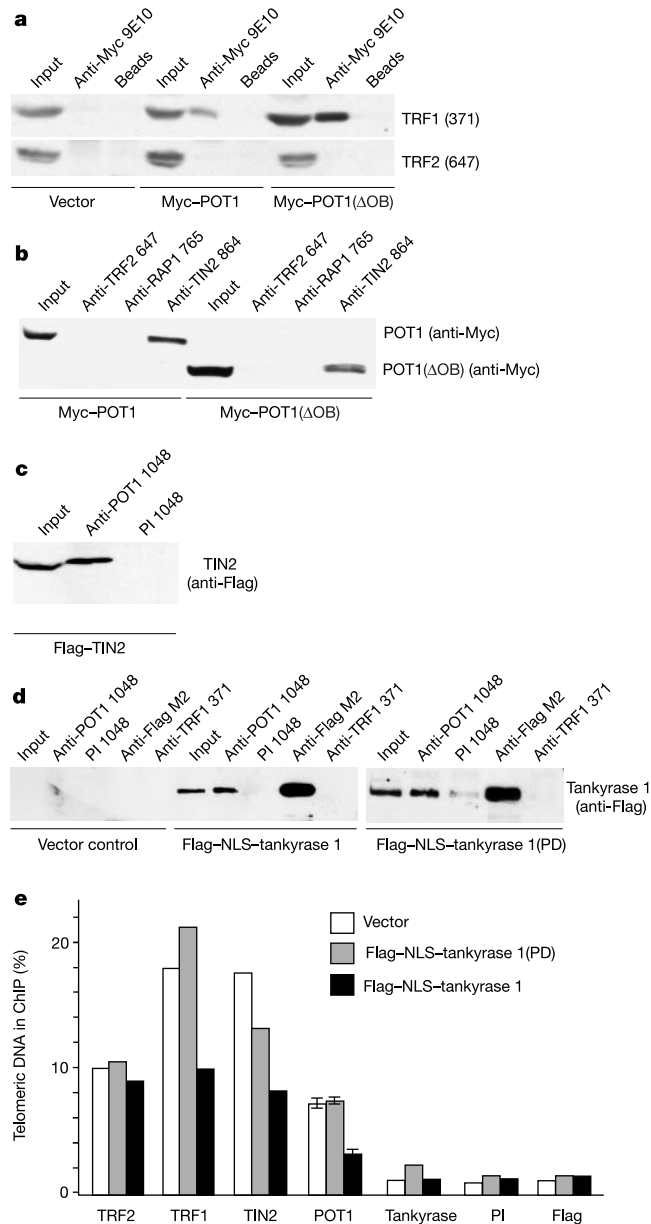


Figure 3 POT1 binds the TRF1 complex and is affected by tankyrase 1.

a-d, Co-immunoprecipitation of POT1 and POT1(ΔOB) with TRF1, TIN2 and tankyrase 1 from HTC75 cells infected with the indicated retroviruses. Immunoprecipitated antibodies are indicated above the lanes. Input, 12% of input extract; PI, pre-immune serum; beads, BSA-blocked beads. Immunoblot antibodies are indicated on the right. **e**, ChIP on HTC75 cells infected with Flag-NLS-tankyrase 1, Flag-NLS-tankyrase 1(PD) or the pLPC vector (as in Fig. 1). POT1 values represent median and standard deviations ($n = 4$). For dot blots see Supplementary Fig. 1.

responsible for the loss of the TRF1 complex from telomeres in cells overexpressing tankyrase 1 in the nucleus⁷. As a control, we used cells expressing the same level of a mutated PARP-dead derivative of tankyrase 1 (tankyrase 1(PD)) that does not remove TRF1 from telomeres (J. Ye and T.d.L., manuscript in preparation; see also ref. 25). As previously observed by immunofluorescence⁷, ChIP data indicated that overexpression of catalytically active tankyrase 1 resulted in diminished telomeric association of TRF1 and TIN2 (Fig. 3e; see also Supplementary Fig. 3). We found that overexpression of tankyrase 1, but not the PARP-dead derivative, reduced by half the amount of POT1 on telomeres as determined by ChIP (twofold reduction, $P = 0.009$; Fig. 3e; see also Supplementary Fig. 3). We note that POT1 is able to interact with both PARP-dead and active tankyrase 1 (Fig. 3d), suggesting that the effect seen with wild-type tankyrase 1 is not simply due to titration by overexpression. TRF2, which serves as a negative control, was not affected by tankyrase 1 (Fig. 3e). Furthermore, the telomeric overhangs were not altered in the cell lines expressing tankyrase 1 or its PARP-dead derivative (Supplementary Fig. 3). Taken together, these data suggest that POT1 is recruited to telomeres through an interaction with the TRF1 complex.

Because the TRF1 complex regulates telomere length, we tested whether POT1 could also affect the steady-state length of human telomeres. Expression of POT1(Δ OB) induced exceptionally rapid telomere elongation (about 230 base pairs per end per population

doubling) in HTC75 cells whereas vector control cells maintained stable telomeres (Fig. 4a, b). This rate of telomere elongation exceeds the growth rate of newly formed telomeres¹¹ and is also faster than the rate of telomere elongation observed in experiments that altered TRF1-mediated length control^{5,7,8}. The POT1(Δ OB) telomeres continued to elongate over at least 150 population doublings, resulting in ultra-long telomeres detectable in genomic blots and in a commensurate increase in TTAGGG repeat signal in dot blots (Fig. 2d). Myc-tagged full-length POT1 did not affect telomere length (Fig. 4b). Neither form of POT1 affected the G-strand overhang signal in HTC75 cells as determined by in-gel analysis (data not shown). The telomere elongation phenotype of POT1(Δ OB) is not attributable to changes in the amount of TRF1 or TIN2 on the telomeres, as ChIP experiments showed that POT1(Δ OB) did not decrease the presence of these proteins (Fig. 2d, e). The finding that these telomeres contained normal amounts of TRF1, yet elongated at a very high rate, indicates that POT1(Δ OB) abrogated the TRF1-mediated inhibition of telomere elongation. This is consistent with POT1 being a downstream effector of the TRF1 pathway and POT1(Δ OB) being defective in this function.

According to the protein-counting model for telomere length homeostasis, longer telomeres should contain larger amounts of the TRF1 complex. We used ChIP of cells with long and short telomeres to test this. HeLa1.2.11 cells have exceptionally long telomeres of

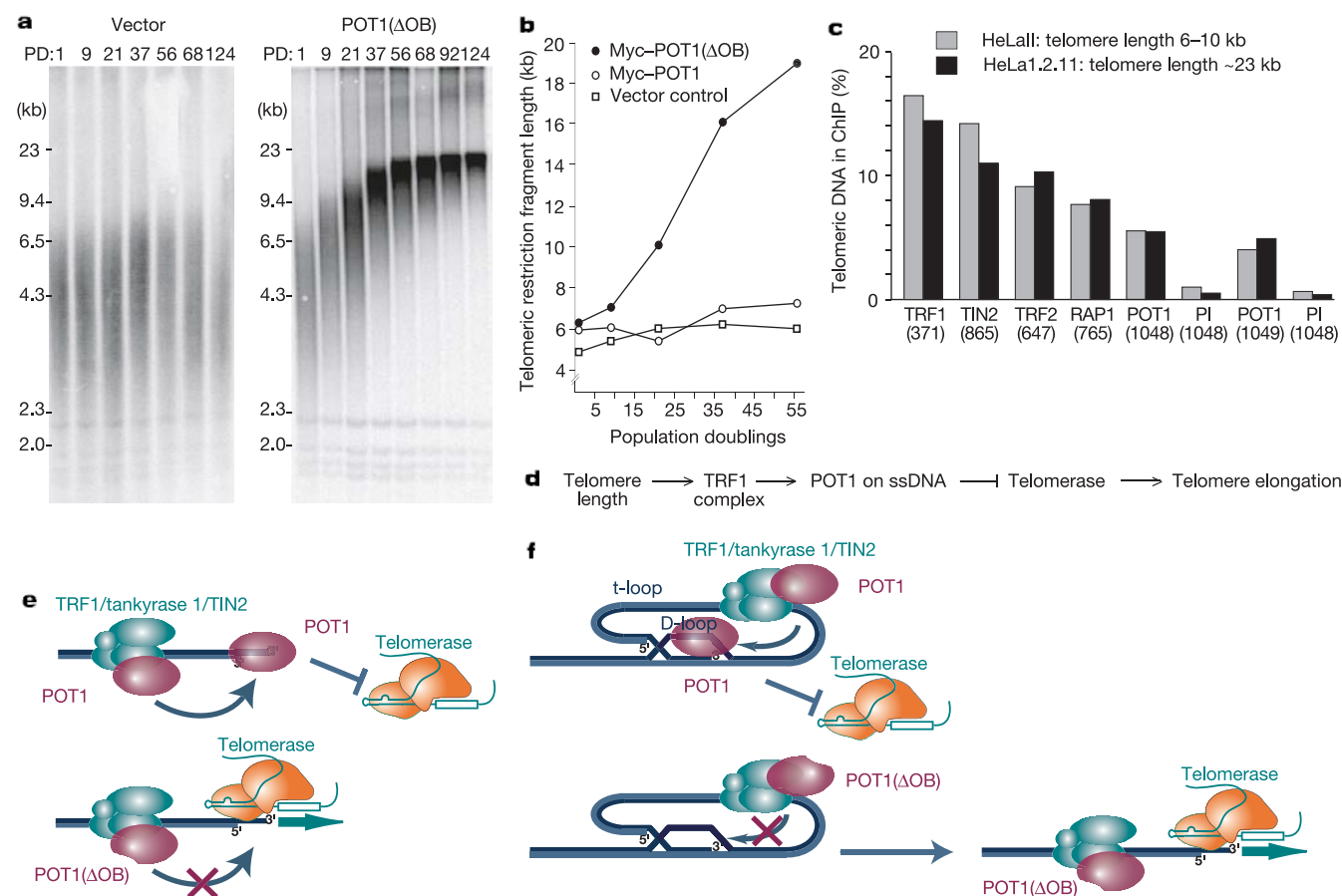


Figure 4 POT1 regulates telomere length. **a**, Genomic blots of telomeric restriction fragments in HTC75 cells at the indicated population doubling (PD) expressing Myc-POT1(Δ OB) or no exogenous protein. **b**, Median telomeric fragment lengths in the indicated cell lines plotted against population doubling. **c**, ChIP yields on telomeres of HeLa1.2.11 and HeLa1 cells. ChIP values are corrected for telomere length as in Fig. 1. As

telomeres of HeLa1.2.11 cells are threefold longer than telomeres of HeLa1 cells, equivalent ChIP of the telomeric DNA in both cell lines indicates that the HeLa1.2.11 ChIP brought down threefold more telomeric DNA. For dot blots see Supplementary Fig. 4b. **d–f**, Models proposing a role for POT1 as a terminal transducer of TRF1 telomere length control. See text for discussion.

approximately 23 kilobases (kb) in length, whereas another HeLa subclone, HeLaII, has canonical telomeres in the range of 6–10 kb (ref. 26). In agreement, dot blots indicated that HeLa1.2.11 had about 2.9-fold higher relative abundance of telomeric sequences (Supplementary Fig. 4a). The telomeres in the two HeLa subclones only differ in the length of the duplex telomeric repeat array (Supplementary Fig. 4c)²⁶; the 3' telomeric overhangs in the two HeLa cell lines are similar (Supplementary Fig. 4c, d). Comparison of ChIPs with antibodies to TRF1 and TIN2 revealed that the fraction of precipitated telomeric DNA in the two HeLa lines was independent of telomere length (Fig. 4c; see also Supplementary Fig. 4b). Thus, the same fraction of telomeric DNA (about 15%) was recovered in TRF1 ChIPs in the two HeLa cell lines. This result is consistent with previous immunofluorescence data⁶ and indicates that longer telomeres contain a higher number of TRF1 complexes. Similarly, the fraction of precipitated telomeric DNA in TRF1 ChIPs was independent of telomere length in two HTC75 cell lines that differed in telomere length by a factor of 3.5 owing to expression of POT1(Δ OB) (Fig. 2d, e). The POT1 ChIP efficiency was also the same in HeLaII and HeLa1.2.11 cell lines (Fig. 4c), suggesting that long telomeres recruited more POT1 than short telomeres. We note that the increased association of POT1 with longer telomeres could be due to more efficient loading of POT1 on single-stranded TTAGGG repeats, or to recruitment along the duplex part of the telomere by protein interaction. The ChIP technique cannot differentiate between these two possibilities owing to the lack of specificity in the crosslinking.

These data demonstrate that the human single-stranded telomeric DNA-binding protein POT1 responds to the telomere length regulatory pathway governed by the TRF1 complex (Fig. 4d). The TRF1 complex binds along the duplex part of the telomere and functions as a measuring device to assess telomere length. For telomere length homeostasis to be effective, information about the length of the telomere needs to be relayed from the TRF1 complex to the single-stranded part of the telomere where telomerase is regulated. Our data indicate that POT1 transduces this information (Fig. 4d–f). The two critical features of POT1 that allow it to relay information about telomere length to the telomere terminus are that it binds to single-stranded telomeric DNA and that its accumulation on telomeres is, in part, regulated by the TRF1 complex.

We predict two possible ways in which POT1 might act to relay telomere length information to telomerase (Fig. 4e, f). The TRF1 complex might recruit POT1 to the telomeric chromatin and facilitate its accumulation on the single-stranded DNA at the nearby telomere terminus. Once located on the single-stranded DNA, POT1, which has a slight preference for 3' ends²⁷ (D.L., H. Parsons, K. Hoke and T.d.L., unpublished data), might sequester the telomere terminus and thus directly block telomerase from elongating the telomeric DNA (Fig. 4e). Alternatively, the recruitment of POT1 to the telomeric chromatin could facilitate its binding to the single-stranded DNA (D-loop) at the base of the t-loop (Fig. 4f). Biochemical studies indicate that POT1 has the ability to bind to single-stranded telomeric DNA in the absence of a 3' end (D.L., H. Parsons, K. Hoke and T.d.L., unpublished data). The binding of POT1 to the D-loop could stabilize telomeres in the t-loop configuration and thus inhibit telomerase by indirect sequestration of the 3' terminus. Both models predict that the POT1(Δ OB) mutant, which lacks the single-stranded DNA-binding activity, will fail to inhibit telomerase, even though POT1(Δ OB) is recruited to the telomeric chromatin by the TRF1 complex (Fig. 4e, f). Thus, POT1(Δ OB) expression will abrogate TRF1-mediated telomere length control, resulting in the observed extensive telomere elongation phenotype. In both versions of the model, control of telomere length is achieved when the larger mass of TRF1 on longer telomeres leads to increased loading of POT1 on the single-stranded telomeric DNA. Depending on the loading of POT1 on the

single-stranded DNA, the probability that telomerase becomes inhibited at the telomere terminus will be increased. □

Methods

POT1 constructs, cell lines and antibodies

A full-length human POT1 complementary DNA (Supplementary Information) was generated by polymerase chain reaction (PCR) and cloned into FastBac and into the pLPC retroviral vector with a Myc epitope tag replacing the start codon. POT1(Δ OB) was constructed by PCR and represents amino acids 127–634 preceded by the Myc epitope tag. GM847 cells were obtained from the American Type Culture Collection. Myc-tagged POT1 and POT1(Δ OB) were retrovirally expressed in HTC75 cells as described²⁸. HTC75 cells infected with retroviruses expressing Flag–NLS–tankyrase 1 and Flag–NLS–tankyrase 1(PD) were provided by J. Ye (J. Ye and T.d.L., manuscript in preparation). All cell lines were grown in DMEM, 15% fetal bovine serum with appropriate antibiotics as described^{21,28}. Antibodies 978 and 979 were affinity purified from rabbit serum to a keyhole limpet haemocyanin-conjugated POT1 peptide (CYGRGIRVLPESNSDQDLKKDLES representing amino acids 271–294 plus a Cys residue). Antibodies 1048 and 1049 were affinity purified from rabbit serum raised against baculovirus-derived POT1. The TIN2 antibody 864 will be described elsewhere (J. Ye and T.d.L., manuscript in preparation). Antibodies to TRF1, TRF2, RAP1 and the Mre11 complex have been described^{5,6,22,23}. 9E10 antibody to the Myc epitope was from Oncogene Biosciences and the M2 Flag antibody was from Sigma.

Chromatin immunoprecipitations

For ChIPs, cells were digested with trypsin, washed with PBS, fixed in 1% formaldehyde in PBS for 60 min at room temperature, washed with PBS, and lysed in 1% SDS, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA at a density of 10^7 cells ml⁻¹. Lysates were sonicated to obtain chromatin fragments <1 kb, and centrifuged for 10 min in a microfuge at 4 °C. Two hundred microlitres of lysate, diluted with 1.2 ml 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, and 150 mM NaCl was supplemented with antibody (typically 20 μ l crude serum or 5 μ l 9E10), mutated overnight at 4 °C, supplemented with 30 μ l protein G Sepharose beads (Amersham; blocked with 30 μ l bovine serum albumin (BSA) and 5 μ g sheared *Escherichia coli* DNA), and incubated for 30 min at 4 °C. Immunoprecipitated pellets were washed with 0.1% SDS, 1% Triton X-100, 2 mM EDTA, pH 8.0, 20 mM Tris-HCl, pH 8.0, containing 150 mM NaCl in the first wash and 500 mM NaCl in the second wash. Further washes were with 0.25 M LiCl, 1% NP-40, 1% Na-deoxycholate, 1 mM EDTA, pH 8.0, 10 mM Tris-HCl, pH 8.0, and with 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. Chromatin was eluted from the beads with 500 μ l 1% SDS, 0.1 M Na₂CO₃. After addition of 20 μ l of 5 M NaCl, crosslinks were reversed for 4 h at 65 °C. Samples were supplemented with 20 μ l of 1 M Tris-HCl, pH 6.5, 10 μ l of 0.5 M EDTA, and 20 μ g DNase free RNase A, and incubated at 37 °C for 30 min. Samples were then digested with 40 μ g proteinase K for 60 min at 37 °C, phenol extracted, and the DNA was precipitated overnight at –20 °C with 1 ml ethanol. The precipitate was dissolved in 100 μ l water, denatured at 95 °C for 5 min, and dot blotted onto Hybond membranes in 2 $\times$ SSC (80% was loaded for the detection of telomeric sequences, and 10% for Alu sequences). Membranes were treated with 1.5 M NaCl, 0.5 N NaOH for 10 min, and with 1 M NaCl, 0.5 M Tris-HCl, pH 7.0, for 10 min. Hybridization with a 800-bp Klenow-labelled TTAGGG probe or an Alu probe was performed as described previously²⁹; membranes were washed four times in 2 $\times$ SSC and exposed overnight to PhosphorImager screen. The quantification of the per cent precipitated DNA was done with the ImageQuant software. All lysates were normalized for cell number. For the total telomeric DNA samples, two 50- μ l aliquots (corresponding to one-quarter of the amount of lysate used in the immunoprecipitations) were processed along with the rest of the samples at the step of reversing the crosslinks. The average of the telomeric signal in two total fractions was taken for the reference value (total DNA), and the percentage of each immunoprecipitation sample was calculated based on the signal relative to the corresponding total DNA signal. Therefore, the calculated ChIP yield takes into account changes in telomere length. Initial control experiments were done to ensure that the amount of antibody and duration of crosslinking were not limiting in the ChIP yields.

Telomere assays and other analyses

Telomere length and overhang assays were as described²⁸. Immunoprecipitations and immunoblotting were performed using standard protocols (Supplementary Information) except for the detection of POT1, which involved guanidine re-naturation (see Supplementary Information for details). Immunofluorescence was performed using previously published procedures⁵ (see Supplementary Information for details).

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Supplementary Information accompanies the paper on www.nature.com/nature.

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Competing interests statement The authors declare that they have no competing financial interests.

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corrigendum

Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1

Emmanuel Treiner, Livine Duban, Seiamak Bahram, Mirjana Radosavljevic, Valerie Wanner, Florence Tilloy, Pierre Affaticati, Susan Gilfillan & Olivier Lantz

Nature **422**, 164–169 (2003).

Owing to mislabelling by the mouse provider, the strain of mice used as a control in the experiment shown in Fig. 4 was not C3H/HeJ but C3H/HeOu. C3H/HeJ have a defect in TLR4-mediated signalling that the other C3H strains do not have. This correction does not affect our conclusions. □

addendum

Non-classical receptive field mediates switch in a sensory neuron's frequency tuning

Maurice J. Chacron, Brent Doiron, Leonard Maler, André Longtin & Joseph Bastian

Nature **423**, 77–81 (2003).

In this Letter, we inadvertently omitted to give the species name, *Apteronotus leptorhynchus* (brown ghost knife-fish), of the weakly electric fish used in our study. □

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that carries out cell counting, apoptosis and viability assessment, and Express — for a variety of applications including protein expression, hybridoma screening, CD marker identification, bead-based supernatant protein/antigen quantification, compound profiling, intracellular protein expression and GPCR expression. In addition, there are three apoptosis assays for the detection of early, mid- and late apoptotic events. The PCA system is expandable so that new applications may be added in the future.

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The Application Control Module (ACM) can be used in conjunction with the NuGenesis Scientific Data Management System to lock down and securely manage Microsoft Excel, Word and PowerPoint files. It provides pharmaceutical and biotechnology companies conducting research and product development with system-wide management of new and existing spreadsheets, documents and presentations. Files are automatically captured, catalogued and kept securely stored, with user authentication via secure user names and password log-in. Files are locked with a complete audit trail of cell and configuration changes, and can be reviewed and signed electronically. Spreadsheets, templates, documents and presentations are easily updated and accessible. File content can be viewed and published in report summaries or project reports and distributed to colleagues.

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Moving targets

Countries often say that to enhance their science base they need to produce more PhDs. But can you end up with too many qualified people? The danger in boosting graduate-school enrolment now is that it could lead to unemployment — or at least longer stints as postdocs — later. That was certainly the situation for US life scientists in the 1990s.

But so far, that seems not to be the case in Finland, according to a survey published by the Academy of Finland last month. The country produced 402 PhDs in 1989, but as part of a concerted effort it has tripled this to 1,224 in 2002. Despite such a dramatic rise, unemployment has not increased. The jobless rate for PhDs in Finland remained below 3% throughout the 1990s, with even lower rates being found in the natural sciences, engineering and medicine.

So is Finland an example or an anomaly? On one hand, it shows how a commitment to building a research-based economy can pay off. In the European Union, Finland is second only to Sweden in terms of PhD density. It is no coincidence that Sweden and Finland also lead the European Union in terms of devoting the highest percentages of their economies to research and development (R&D). On the other hand, Finland is an anomaly in that it, like Sweden, is a relatively small country, with a fairly indigenous R&D population.

But other countries may soon need to consider Finland's approach. For example, in Britain, fewer students are taking chemistry. And the United States may need to boost its own internal science production as it is putting up barriers to foreign scientists, who traditionally make up the country's R&D needs. The only danger in setting targets is that, by the time a need is identified, it is likely that the need has already changed.

Paul Smaglik

Naturejobs editor



Contents

MOVERS

A first for the Karolinska Institute; fresh perspectives for US engineering; Danish crystallographer heads for France; and more

p1022

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Information on the
scientific job market

FOCUS

SPOTLIGHT

RECRUITMENT

ANNOUNCEMENTS

EVENTS

Transitions

X Göran Ando, head of research and development at US drug group Pharmacia until it was bought by Pfizer last month, has become chief executive of Celltech, Britain's largest biotech company. The move comes in preparation for the firm's impending acquisition of Oxford GlycoSciences. Ando's predecessor at Celltech, **Peter Fellner**, is to become the company's chairman.

X Brian Boyle, director of the Anglo-Australian Observatory in Sydney, will take up the directorship of the Australia Telescope National Facility in July.

X Photonics researchers **David Cotter**, **Andrew Ellis**, **Robert Manning** and **Paul Townsend** are relocating from the Corning Research Centre in Ipswich, UK, to University College Cork, Ireland, as part of an €11.1-million (US\$13-million) grant from Science Foundation Ireland.

X This July, **Anindya Dutta** will move from Brigham and Women's Hospital in Boston to the University of Virginia, Charlottesville, to become professor of biochemistry and molecular genetics and professor of pathology.

X Prabhavathi Fernandes has joined the supervisory board of GPC Biotech, a biotechnology firm based in Martinsried, Germany. She most recently served as chief executive at Ricerca Biosciences in Concord, Ohio.

X London-based SR Pharma has appointed **David Hill** as chief executive and board member. Hill was previously chief executive of Sedac-Therapeutics, an immunotherapy-development company based in Lille, France.

MEDICINE

Harriet Wallberg-Henriksson can claim a couple of firsts: she was the first female dean of the Karolinska Institute's medical faculty and the first female head of the Swedish Scientific Council for Medicine. Last month, she added another to her list — first female president of the Karolinska Institute in Stockholm. The irony is, she never set out to be a trail-blazer for her gender.

In the mid-1990s, she was asked to serve as deputy head of the Swedish medical research council. As it was a part-time job, she kept her position as professor of physiology and gritted her teeth to do battle with bureaucracy. "To my big surprise I really liked doing research administration," Wallberg-Henriksson says. She will replace **Hans Wigzell** at the Karolinska Institute next January, after he finishes his second four-year term.

ADMINISTRATION

Reflecting on his professional journey, which has taken him from New Zealand to Britain and from University College London (UCL) to the University of Cambridge and back, **Malcolm Grant** considered how much of his movement was by design. "Do people plan their careers?" he asks. "The answer is, absolutely not." Grant, who will leave his position as pro-vice-chancellor of the University of Cambridge in October to become UCL's provost, says that his origins as a law professor led somewhat serendipitously to his current incarnation as an administrator.

His training in environmental law led to his first UCL post as a law professor and his later role as chair of the UK Agriculture and Environment Biotechnology Commission, which advises the government on the implications of biotechnology. That involvement will help him at UCL. "In that capacity, I've had quite a lot of encounters with biotechnology and research," he says.

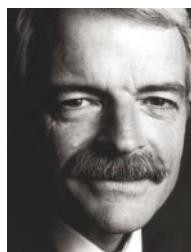
Grant sees his primary challenges at UCL in terms of fixing old buildings and creating new ones on a limited budget, as well as finding ways to pay faculty members more — something he says is necessary to be competitive. The erosion of staff salaries at leading UK universities is "nothing short of a scandal", Grant says.

BASIC RESEARCH

John Brighton has decades of experience in steering the research directions of individual universities. Last month, he began guiding engineering funding for



Harriet Wallberg-Henriksson



Malcolm Grant



John Brighton



Sine Larsen

the US National Science Foundation (NSF). Brighton, who was previously provost of National-Louis University in Evanston, Illinois, is now head of the NSF's directorate for engineering. Before his stint at National-Louis, he was provost and professor at Pennsylvania State University, where he was also dean of the engineering college. Before that, he held faculty positions at the Georgia Institute of Technology, Michigan State University, Carnegie Mellon University, and Purdue University.

In those positions, Brighton served on the other side of the NSF review process, helping other faculty members to submit research proposals for grants. Now, he is looking forward to deciding what broad areas of basic engineering should be funded. That prospect attracted him to the job. "I thought it might be interesting, meeting with the top people of the country regularly in particular disciplines and talking to them about what's important, what's emerging, what we should be emphasizing," says Brighton. He is especially excited about identifying and funding young, up-and-coming researchers and encouraging more collaborative work nationwide.

STRUCTURAL BIOLOGY

This month, **Sine Larsen** left her vice-dean position in the natural-science faculty at the University of Copenhagen to become research director at the European Synchrotron Radiation Facility (ESRF). Although she has moved to the ESRF's home in Grenoble, France, she plans to keep her structural biology group in Copenhagen intact.

For Larsen, the decision to move to France marks a dramatic change. She has been at the University of Copenhagen since returning from a postdoc at the Massachusetts Institute of Technology in 1974. She acquiesced to the headhunting partly because her children had grown up, so moving wouldn't disrupt her family. "It should be a good time to take new challenges," she says.

She doesn't speak French, but is looking forward to learning. She is also looking forward to working with **Francesco Sette**, the ESRF's other research director. Larsen brings a chemistry background to the synchrotron, whereas Sette provides physics skills. In addition, Larsen says that she will retain her position as general secretary and treasurer in the International Union of Crystallography.

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